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(54) Title: METHODS AND APPARATUS FOR DETECTING THE EFFECT OF CELL AFFECTING AGENTS ON LIVING CELLS

(57) Abstract

Methods and apparatus are disclosed for detecting the effects of cell affecting agents on living cells. The method steps include providing living cells that are retained in a micro flow chamber. The micro flow chamber is adapted for either continuous or intermittent flow of solutions or suspensions in intimate contact with the cells. The solutions or suspensions, which contain a cell affecting agent, are then flowed in intimate contact with the cells and at least one effect of the cell affecting agent on the cells is measured by an appropriate detecting means, which is operatively associated with the micro flow chamber.

- 1 -

METHODS AND APPARATUS FOR DETECTING THE EFFECT
OF CELL AFFECTING AGENTS ON LIVING CELLS

BACKGROUND OF THE INVENTION

Field Of The Invention

5   This application is a continuation-in-part of copending application Serial No. 260,521, filed October 21, 1988.

The present invention relates to methods for detecting the effects of cell affecting agents on living cells and to apparatus adapted to the practice of such methods. Solutions or suspensions of

10   cell affecting agents are flowed over cells and the effects of these agents are measured.

Description Of The
Background Of The Invention

Studies of the effect of various cell affecting agents on living

15   cells have been reported in the literature. See. e.g.. Meisner, H. and Tenny, K. (1977) "pH as an indicator of free fatty acid release from adipocytes," J. Lipid Research, 18:774-776; Nilsson, N. and Belfrage, P. (1979) "Continuous monitoring of free fatty acid release from adipocytes by pH-stat titration," J. Lipid Research 20:557-560;

20   Reuss, L., Weinman, S. and Constantin, J. (1984) "H^+ and HCO^-_3 transport at the apical membrane of the gallbladder epithelium," pp. 85-96 of Forte, J., Warnock, D. and Rector, F. Jr. (eds.) Hydrogen Ion Transport in Epithelia, Wiley - Interscience; Zeuthen, T. and Machen, T. (1984) "HCO^-_3/CO_2 stimulates NA^+/H^+ and Cl^-/HCO^-_3 exchange

25   in Necturus gallbladder," pp. 97, 108 (ibid.); Handler, J.S., Preston, A.S. and Steele, R.E. (1984) "Factors affecting the differentiation of epithelial transport and responsiveness to hormones," Federation Proceeding 43:2221-2224; and Simmons, N.L., Brown, C.D.A. and Rugg, E.L. (1984) "The action of epinephrine on

30   Madin-Darby canine kidney cells," Federation Proceedings, 43:2225-2229. These references disclose the detection of changes in pH and other electrical potentials by the addition of cell affecting agents

- 2 -

to cells disposed in a relatively large amount of medium, i.e., a
bulk medium. A disadvantage of these techniques is that the pH and
other electrical potential measurements are taken from the bulk
medium and do not necessarily reflect the actual values immediately
adjacent to the cellular membranes of the living cells. Also, the
high ratio of bulk volume to cell volume inevitably dilutes the
effects of the cells on the properties of the extracellular medium.
Accordingly, sensitivity is lost or greatly reduced.

Photoresponsive sensors for measuring biochemical systems are
disclosed in various patent documents owned by the assignee of the
present invention. See, e.g., U.S. Patents Nos. 4,591,550 (Hafeman
et al.) and 4,704,353 (Humphries et al.); and European Patent
Application No. 213,825 (Hafeman et al.). U.S. Patent No. 4,519,890
discloses a flow pH chamber. These patent publications disclose the
use of microorganisms to measure changes in the environment of the
solution to be measured. There is no disclosure in these
publications of cells in micro flow chambers used to measure of the
effects of cell affecting agents. See, also, U.S. Patents Nos.
4,737,464 (McConnell et al.) and 4,741,619 (Humphries et al.), which
are likewise owned by the assignee of the present invention.

Various ways of using fluorescence to measure extracellular
effects of living cells and analytes are disclosed in the literature.
See, e.g., Briggs et al. (1985) "Fiber Optic Probe Cytometer" J.
Immunological Methods, 81:73-81; Hafeman et al. (1984) "Superoxide
Enhances Photo Bleaching During Cellular Immune Attack Against
Fluorescent Lipid Monolayer Membranes" Biochemica Biophysica Acta
772:20-28; and U.S. Patents No. 4,560,881 and 4,564,598.

- 3 -

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for detecting the effects of cell affecting agents on living cells with greater accuracy and precision.

It is an additional object of the present invention to provide methods for detecting the effects of cell affecting agents on living cells that avoid the use of large volume, bulk media.

It is a specific object of the present invention to provide methods for detecting an effect of a cell affecting agent on living cells by: (a) providing living cells that are retained in a micro flow chamber adapted for continuous or intermittent flow of a solution or suspension containing the cell affecting agent in intimate contact with the cells so that the amount of cell affecting agent in contact with the cells may be controlled; (b) flowing a solution or suspension containing the cell affecting agent in intimate contact with the cells, thereby producing a cell mediated extracellular effect or change in pH, redox potential, cell surface potential or trans-cellular potential; and (c) measuring the effect of the cell affecting agent by a means for detecting pH, redox potential, cell surface potential or trans-cellular potential that is operably associated with the micro flow chamber.

It is a further object of the present invention to provide apparatus particularly adapted to practice the inventive methods.

In a preferred embodiment of the invention, the cells may be retained in the micro flow chamber by spontaneous or natural adhesion of the cells to the internal surface of the flow chamber or on a porous membrane or microcarrier contained in the flow chamber. Alternatively, the cells may be retained in the micro flow chamber by means of a binding agent that is biologically compatible with the cells. A preferred example of such a binding agent is agarose.

In another preferred embodiment of the present invention, the living cells may be retained in the micro flow chamber by providing the micro flow chamber with a surface having a plurality of wells or depressions that act to physically trap the cells on the surface of the micro chamber, preferably by gravitational sedimentation. These wells should be of a sufficient width and depth such that the cells

- 4 -

remain in the micro flow chamber during ordinary flow rates. The
cells may then be removed from the trapping wells by any appropriate
means, including the use of high flow rates through the micro flow
chamber that wash the cells out of the wells or by inversion of the
5 chamber to dislodge the cells from the wells into the flow stream.
In an alternative embodiment, the cells may be retained in the flow
chamber by trapping them within a compartment of the flow chamber
separated by a porous membrane.

Some features of the preferred geometry of the micro flow
10 chamber used in the present invention are common to most applications
of the device. Chief among these is the volume-to-surface ratio in
the chamber, which controls the maximum concentration of adherent
cells. The pH change per proton excreted by a cell is inversely
proportional to the cell concentration and, therefore, to the volume-
15 to-surface ratio. For the planar slab geometry of the chamber as
described in Figures 1-4, this ratio is simply the chamber height,
typically $100\mu m$.

The design tradeoff involving volume-to-surface ratio is
principally one of sensitivity versus ease of fluid handling. With
20 our present preferred apparatus, sensitivity becomes seriously
degraded for ratios above approximately $200\mu m$. For thicknesses below
approximately $50\mu m$ we anticipate significant fluid-handling problems
that might offset sensitivity gains. However, instrumental
improvements, such as chambers micro-machined into the sensor
25 surface, might allow an integration of fluidics and chamber on a much
smaller scale, permitting the analysis of one or a few living cells
at ratios of $10-50\mu m$.

The preferred volume of the micro flow chamber used in the
present invention will depend to some extent on the intended
30 application of the device. For example, if the number of cells
available is a limitation, then sub-microliter chamber volumes are
preferred (e.g., $1 \ mm^2$ surface area and $100\mu m$ height, or 100nL,
containing about 10^3 cells). For other applications involving a
larger number of cells, chamber volumes on the microliter or even
35 milliliter scale can be envisioned (surface areas on the order of
square centimeters, with about $10^5 \ cells/cm^2$, but still maintaining

- 5 -

approximately 100μm chamber height). Thus, the preferred micro flow chamber has both a small total volume achievable in the chamber and/or a small height or volume-to-surface ratio.

The type of micro flow chamber that is preferably used in the
5   inventive method is of the type disclosed in U.S. Patent No. 4,591,550, the disclosure of which is incorporated herein by reference. An argon and/or helium/neon laser may be used as a source of energy in place of the light emitting diodes disclosed in U.S. Patent No. 4,591,550. Alternatively, the micro flow chamber useful
10   in the present invention may be of the type disclosed in pending U.S. patent application serial no. 876,925, filed June 20, 1986, which is owned by the assignee of the present invention, the disclosure of which is incorporated herein by reference. Most preferably the micro flow chamber to be used in the present invention includes a silicon
15   semiconductor electrode on or near which the living cells are retained. By means of this electrode, the various electrical effects caused by the cell affecting agent may be detected or measured. The micro flow chamber to be used in the present invention should preferably provide for both intermittent and continuous flow of
20   solutions or suspensions, since either intermittent or continuous flow of solutions or suspensions may be used in practicing the present invention.

The present invention may be used in conjunction with either eukaryotic or prokaryotic cells, so long as the particular cells are
25   capable of being retained in the micro flow chamber. Genetically transfected cells may also be used. In addition, a wide variety of cell affecting agents may be used, including irritants, drugs, toxins, other cells, receptor ligands, receptor agonists, immunological agents, viruses, pathogens, pyrogens, and hormones.
30   A wide variety of effects caused by the cell affecting agents may be detected or measured according to the present invention. Preferred effects include the pH, redox potential, and other electrical properties of the solution or suspension that flows in intimate contact with the living cells in the micro flow chamber,
35   such as cell surface potential and trans-cellular potential. These effects may be measured or detected by a variety of conventional

- 6 -

means. For example, pH can be detected by measuring the fluorescence or absorbance of a pH sensitive dye such as fluorescein or phenol red in the extracellular medium or fixed to a part of the chamber. In a similar manner other dyes can be used to detect redox potential.

5   The present invention also includes methods of identifying microbes, methods of screening for the activity of drugs, methods for detecting toxic substances and methods for detecting intercellular reactions.   In these various methods, solutions or suspensions containing the desired cell affecting agent are flowed in intimate

10   contact with the living cells retained in the micro flow chamber. The effect(s) of the cell affecting agent on the cells are then measured and provide the means by which bacteria may be identified, drugs screened, and toxins and intercellular reactions detected.

      The present invention has several advantages over other methods

15   of measuring metabolic activity, such as oximetry and microcalorimetry.   Specifically, the present invention possesses rapid measurement time, enhanced sensitivity, noninvasiveness, generality and the potential for further miniaturization and integration.

20   Certain preferred embodiments of the present invention are discussed below in more detail in connection with the drawings and the detailed description of the preferred embodiments.   These preferred embodiments do not limit the scope or nature of the present invention.

- 7 -

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1-3 represent schematic cross-sectional views of micro flow chambers useful in practicing the preferred inventive methods.

FIGURE 4 represents a schematic top perspective view of a micro flow chamber useful in practicing the preferred inventive methods.

FIGURE 5 represents a schematic diagram of apparatus useful in practicing the preferred inventive methods.

FIGURE 6 represents a schematic diagram of a degasser useful in practicing the preferred inventive methods.

FIGURE 7 represents a schematic side view of adherent cells located in a micro flow chamber.

FIGURE 8 represents a schematic side view of cells retained in wells in a micro flow chamber.

FIGURE 9 represents a diagram of a circuit useful in the present invention.

FIGURE 10 represents a typical determination of cellular metabolic rates that may be made by use of the present invention.

FIGURES 11-23 represent the experimental results obtained in connection with Examples 1, 2, 4, 6, 9, 11 and 17.

FIGURE 24 represents results of exposing gram negative bacteria to an antibiotic which has an effect and one that has no effect on the gram negative bacteria.

FIGURE 25 represents a schematic plan view of wells for retaining cells useful in practicing the preferred inventive methods.

FIGURES 26-46 represent the experimental results obtained in connection with Examples 19-23.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Referring to FIGURE 5, the instrumental setup of the preferred embodiment preferably consists of one or more syringe drives or pumps, a degassing chamber, a selection valve or injection loop valve, a flow chamber, a reference electrode reservoir, and the associated electronics required for the silicon semiconducting electrode sensor and data processing.

The syringe drives provide the solutions or suspensions (i.e., the medium) to the flow chambers at controlled flow rates. Both the

- 8 -

flow rate and the on/off cycle of the syringe drives can be controlled by means of a computer during the data acquisition phase. Alternatively. other types of pumps, such as peristaltic pumps, can be used in place of the syringe drives.

Referring to FIGURE 6, the degassing chamber consists of the length of silicone rubber tubing through which the solutions or suspensions pass. The outside of the tubing is maintained in a reduced pressure air atmosphere (approximately 2/3 atmospheric pressure). A degassing chamber is preferred because the solutions or suspensions in the syringes and tubing are at room temperature and the flow chamber is commonly at 37°C. As the room temperature medium reaches the flow chamber it warms to 37°C resulting in the formation of bubbles in the chamber. Bubbles in the flow chamber interfere with measurements in several ways. They can cause a high resistance path to the reference electrode resulting in an increase in noise in the photocurrent signal. They can also alter the aqueous volume in the chamber thus changing the buffer capacity of the chamber. For a given cellular metabolic rate, the rate of change of pH in the chamber will vary with the number and size of the bubbles. Bubbles form in the chamber and grow with time. When they reach a certain size they dislodge and are carried away in the flowing stream. This can create low frequency noise in the buffer capacity of the flow chamber.

The selection valve allows the user to direct the flow from one of two syringes into the flow chamber. An alternative is an injection loop valve. This allows the user to inject a bolus of solution or suspension into the flow stream without interrupting the flow. Typically when the injection loop valve is used, one syringe is loaded with medium and used to perfuse the stream by means of the injection loop. The volume of the agent solution or suspension and the time of exposure of the cells to the agent can be varied by varying the injection loop size and the flow rate of the perfusion stream.

FIGURES 1-4 illustrate a preferred micro flow chamber 1 having a silicon sensor 2 with an inlet port 3 and an outlet port 4. In FIGURE 1, cells 5 are adhered in a monolayer to the upper surface of

silicon sensor and the response on the silicon sensor is modulated by
laser light 6. Alternatively, the cell can be retained adjacent the
silicon sensor by a permeable membrane. In operation, a solution
containing a cell affecting agent enters through inlet port 3 and
5   flows over cells 5 in the micro flow chamber 1. The solution exits
outlet port 4 where the exiting solution is in electrical contact
with a reference electrode. The local response on the upper surface
of the silicon sensor 2 is modulated by laser light 6 and measured.
FIGURE 2 illustrates wells 7 that trap or retain therein cells during
10   slow flow rates and from which the cells may be flushed out of the
flow chamber during fast flow rates.

The flow chamber is preferably a thin channel bounded on the
bottom by the silicon sensor 2, and on the top by an indium-tin-oxide
(ITO) coated glass cover slip 8. Adherent cells may also be grown on
15   the surface of the cover slip instead of the silicon semiconductor
electrode. The spacing between the silicon sensor and the cover slip
is approximately 100 μm. The spacing between the sensor 2 and the
cover slip 8 may be achieved by means of a Teflon spacer 14 that has
an appropriate channel cutout that forms the flow chamber. A
20   controlling electrode 13, preferably a platinum wire, penetrates the
plastic housing of the flow chamber to make electrical contact with
the ITO controlling electrode. Electrical contact with the silicon
sensor is made via the metal baseplate 9 of the flow chamber. Teflon
inlet and outlet tubes, 3 and 4, penetrate the plastic housing and
25   allow medium to flow through the flow chamber. The outlet tube 4
terminates in a reservoir that contains a Ag/AgCl reference
electrode. Thus the outlet tube 4 acts as a salt bridge to allow
measurement of the potential of solutions or suspensions in the flow
chamber. The entire flow chamber is mounted on a hollow metal
30   support that is maintained at constant temperature, typically 37°C,
by a temperature controlled circulating water bath 10. The cover
slip 8 is removable and may be maintained in operative position by
means of removable silicon rubber gaskets 11 and retaining members
12.

35   Two different instruments may be preferably used for the purpose
of illuminating the silicon sensor to generate a suitable

photoresponse: a laser instrument and a light emitting diode (LED) instrument.

In the laser instrument, the flow chamber is mounted on a light microscope stage. The beam from a 150mW argon ion laser is directed through a 10kHz mechanical chopper and into a beam expander that generates a beam approximately 2.5 cm in diameter. The beam then passes through a polarizing filter (variable attenuator) and is directed by mirrors through a quadruple knife edge adjustable aperture into the barrel of the microscope. Alternatively, in one version of the apparatus, the chamber is mounted on a microscope stage and the beam from a 10mW HeNe laser is projected through an epi-illumination system onto the sensor. In these configurations, one can see the cells in the flow chamber through the microscope and can direct a square or rectangular probe beam of any desired size to any region of the silicon surface in the flow chamber. This is particularly useful when nonconfluent cell cultures are used. It allows one to direct the probe beam to regions of greatest cell density. In addition one can control for instrumental drift by directing the beam to regions where no cells are visible.

In the LED instrument, two flow chambers are mounted side by side on a temperature controlled plate. For illumination, four fiber optic light guides for each chamber penetrate the temperature controlled plate to illuminate the silicon sensor from underneath (the side opposite the surface in contact with the medium). The four fibers are aligned so as to have one near the inlet tube, one near the outlet tube, and two equally spaced between the inlet and the outlet. This instrument is preferably used with cells that grow to a reasonably consistent and uniform density. However, it still gives some latitude in picking regions of greatest cell density. One end of the optical fibers butts up against the silicon sensor and the other end is coupled to an infrared LED. Each fiber is 1.5mm in diameter. Data can be collected from eight sites (four for each of the two chambers) simultaneously every four seconds.

There may be three primary electrical connections to the chamber: (1) a controlling electrode connected to the indium-tin oxide coating on the cover slip via a thin platinum ribbon or wire;

- 11 -

(2) a Ag/AgCl reference electrode in an external well that measures the potential of solution in the flow chamber via a salt bridge comprising the chamber's outlet tube; and (3) an ohmic connection to the base of the silicon chip. A personal computer with a custom

5 circuit board may handle the acquisition, analysis, and display of data in ways that are well known in that art.

A preferred circuit for use in the present invention is disclosed in FIGURE 9. The sensor electrode system is shown comprising a silicon sensor, a reference electrode (R.E.) and a

10 controlling electrode (C.E.). The controlling electrode maintains the solution at an arbitrary potential with respect to ground via a low impedance contact to ground. The arbitrary potential is defined by the surface potential at the electrode/solution interface and the various contact potentials between the electrode material and ground.

15 The reference electrode is of the salt bridge type, and thus measures the potential of solution independent of solution composition. The purpose of the electronic circuit is twofold. First, it allows for varying the potential from the solution to the electrical contact to the silicon. Second, it converts a photogenerated alternating

20 current to an alternating voltage (the output signal). U2A is configured as a conventional voltage follower whereby the reference electrode potential is buffered. U1 is a digitally controlled bipolar current source. A connection between pins 18 and 19 forms a current loop. The sign and magnitude of the current in this loop is

25 controlled by the digital word applied to pins 1 through 13. The potential on pins 18 and 19 floats with respect to ground. As configured in the circuit, the combination of U1 and U2A allows the voltage at pin 5 of U2B to be offset from the voltage at pin 3 of U2A. The sign and magnitude of this voltage offset are determined by

30 the digital word applied to U1, and the values of R1 and R2. U2B is used both as a voltage follower to apply a known offset voltage through R3 and R4 to the silicon, and as a current to voltage converter to convert the alternating photocurrent to an alternating voltage at pin 7. The signal gain of U2B is determined by R3. R4 is

35 used to match the impedance of the circuit to that of the sensor. The voltage from silicon to solution is swept by applying a series of

- 12 -

incrementally increasing words to U1. The signal amplitude (alternating photocurrent) is read out at U2B pin 7 as a function of potential applied from solution to silicon.

Living cells may be adhered to the chamber in a variety of ways. As shown in FIGURE 7, naturally adherent cells may be grown either on the surface of the silicon sensor or the cover slip in an incubator or both. The flow chamber is assembled while keeping the cells moist. Flow is established quickly to keep the cells from over acidifying the medium or consuming all of the oxygen in the chamber. For non-adherent cells, the cells may be mixed with an agarose solution at 37°C and plated onto the surface of the silicon sensor or cover slip in an approximately 50 μm thick layer in a humid atmosphere. The silicon sensor is then refrigerated for about 15 min. to solidify the agarose. The flow chamber is then assembled and used.

Collagen and gelatin may also be used in conjunction with or in place of agarose. Fibronectin, chondronectin, laminin or other similar substances may optionally be used in adhering the living cells to the surface of the micro flow chamber. Alternatively, the living cells may be dispersed on or in biologically compatible microcarrier beads.

In another embodiment, cells may be grown on or in a porous membrane. This membrane may be inserted into the flow chamber between the controlling electrode and the sensor surface so that culture medium flows along at least one side of the membrane. Some cells, such as epithelial cells, form a monolayer with differential apical and basolateral surfaces. In such cases the properties of both sides of the cell sheet can be studied separately by controlling which side of the membrane is closer to the sensor surface.

The generally preferred inventive method may be practiced as follows. The chamber is assembled with living cells retained therein and attached to the fluidics in the instrument. Flow (typically 100μL/min) of a low buffer capacity solution or suspension is established and the signal from the sensor is allowed to stabilize. Normal cell-culture medium may be used, including serum if desired, except that bicarbonate and buffers such as HEPES should be excluded.

- 13 -

Some drift is seen initially due to warming of the chamber and equilibration of the chamber with the new medium. Once stable, flow in the chamber is halted and the silicon sensor signal is monitored. A decrease in potential reflects the decrease of pH in the chamber due to cellular metabolism. The rate or slope of pH decrease is a measure of the metabolic rate of the cells. The pH is allowed to drop far enough to obtain enough data points to accurately determine the slope of the line (within a few percent error). Typically this reflects a pH drop of approximately 0.1 to 0.5 pH units and requires approximately 1 to 4 minutes. Flow is then restarted and the pH returns to the initial value. This sequence is repeated until reproducible slopes are obtained. A typical determination of metabolic rate is shown in FIGURE 10.

In another preferred embodiment, medium is flowed continuously over the cells and the potential is measured at a plurality of sites. If one site is located upstream of the direction of flow from another and there are intervening cells, there will be a pH difference between the two sites with the upstream site being less acidic. This pH difference is due to the metabolic action of the cells on the medium as it traverses the space between the sites. The magnitude of this pH difference, as detected by the silicon semiconductor electrode, is a measure of the metabolic rate of the cells located between the two sites.

For testing cell affecting agents, two strategies may be advantageously employed. In one approach, two syringe drives are used. First metabolic rates are determined as described above with a control medium. Then the syringe drive with the control medium is turned off and a syringe drive with the same medium plus the cell affecting agent is turned on. An appropriate selection valve directs the flow from either syringe drive to the flow chamber. Metabolic rate measurements are then taken in the presence of the cell affecting agent. The flow can be switched back to the control medium to observe recovery of the cells from the effect of the cell affecting agent.

In a second approach, an injection loop valve is used. Metabolic rates are determined as described above. Once they are

- 14 -

stable, the cell affecting agent is introduced into the injection loop, and the valve is turned such that the cell affecting agent is introduced into the flowing medium stream. Metabolic rates can then be measured in the presence of the cell affecting agent and can be

5   followed after the agent is washed out of the chamber. This technique is particularly suited for testing for cell responses to brief exposure to cell affecting agents.

If the living cells to be used are not naturally or spontaneously adherent to a surface of the micro flow chamber, means

10   of retaining the cells in the chamber must be used. Three desirable ways of achieving this result are the provision of wells capable of physically retaining the cells, the provision of a compartment in the micro flow chamber that retains the cells by means of a membrane that is porous or permeable to the cell affecting agent in the solution or

15   suspension, and the use of biologically compatible adhering agents.

In the embodiment employing wells, as shown in FIGURE 8, the floor of the flow chamber contains the wells. The wells may be circular in cross-section or may be trenches aligned perpendicularly to the direction of flow. The smallest horizontal dimension of the

20   wells should be at least one cell diameter, preferably no more than several cell diameters. The depth of the wells can be several cell diameters. Wells $50\mu m$ in diameter and $50\mu m$ deep are acceptable. The use of wells to retain the cells is well adapted for the analysis of multiple batches of cells by employing the following cycle of

25   operations.

The first step is loading cells into the wells. At sufficiently slow flow rates cells will sediment into the wells and remain trapped therein.

Next, the cellular behavior is analyzed. The composition of the

30   medium flowing over the wells can be altered according to the desired analytical protocol. Compounds from the medium reach the cells in the wells by a combination of diffusion and convection.

Finally, the cells are flushed out of the wells. At sufficiently high fluid flow rates, vortex behavior in the wells

35   becomes strong enough to cause the cells to be resuspended and to be

- 15 -

swept out of the wells and out of the chamber. This clears the chamber for the next loading of fresh cells.

There are at least two important advantages to the well method. First the method of immobilization is nonperturbing. Second, the system is easily automated to provide repetitive analyses of fresh batches of cells in the same micro flow chamber.

In the embodiment employing biologically compatible adhering agents, non-adherent cells may be retained in the flow chamber in a very thin (preferably 50μm thick) layer of agarose covering the floor of a flow chamber having a total depth 100μm and surface area about one cm^2. Medium flows through the 50μm gap remaining between the ceiling of the chamber and the top of the agarose layer.

The thinness of the agarose layer permits rapid and virtually unimpeded access of the immobilized cells to the components of the medium flowing over the agarose. Transport between the cells and the flowing medium is diffusive. Diffusion times on the order of one to ten seconds are to be expected for most solutes.

The cell retaining wells are preferably formed on the surface of the silicon semiconductor electrode or sensor. They may be formed as indentations in the surface of the electrode or by application of a grid structure on top of the upper surface of the electrode. The grid structure is preferably formed of an electrically compatible semiconducting material, i.e. silicon, but may be formed of electrically neutral materials. For example, a nylon mesh comprised of 25μm diameter threads woven to produce 25μm square holes may be glued to the surface of the silicon semiconductor electrode with a spray adhesive. The adhesive forms widely dispersed fine droplets on the silicon electrode and therefore only attaches the nylon mesh to the surface at a few discrete points. In this manner, the nylon mesh may be attached to the silicon electrode without totally coating the silicon electrode with adhesive and thereby destroying the photoresponse of the electrode.

With adherent cells, unpatterned silicon surfaces may be fabricated by first growing a thin (30 nm) thermal SiO_2 film, followed by a deposition of 100 nm of Si_3N_4 by low-pressure chemical vapor deposition. The first step in the fabrication of the

- 16 -

micromachined sensor for use with non-adherent cells is to grow a $1\mu m$ thick SiO_2 layer by steam oxidation, followed by photolithographic patterning to form openings where the cavities will be located. The cavities are then anisotropically etched in an SF_6/C_2ClF_5 plasma,

5   producing cavities with sidewalls at an angle of $>85°$ from the horizontal. The final steps in the process are the growth of the thin oxide and the nitride deposition, as in the unpatterned case. The micromachined sensors thus have a thick oxide layer on the areas between cavities, effectively deactivating the sensing capability

10   there. The active sensing surfaces are only the inside walls and floor of the cavities, improving the signal-to-noise performance of this version of the device.

The device detects the surface potential at the interface between the electrolyte and the Si_3N_4 insulator. The presence of

15   protonatable silanol and amine groups on this surface ensures that this potential depends on pH in a Nerstian fashion. The pH is detected only at portions of the interface corresponding to illuminated regions of silicon.

The present invention may be used with a wide variety of

20   prokaryotic and/or eukaryotic cells. Examples of such cells include human keratinocytes, murine L fibroblastic cells, canine MDCK epithelial cells, hamster BHK fibroblastic cells, murine CTLL lymphocyte cells, tumor cells and bacteria. In general, any living cells, including transfected cells, that can be successfully retained

25   within a micro flow chamber may be used.

The present invention may be used with a wide variety of cell affecting agents. Examples of such cell affecting agents include irritants such as benzalkonium chloride, propylene glycol, methanol, acetone, Na dodecyl sulfate, hydrogen peroxide, 1-butanol, ethanol,

30   and DMSO; drugs such as valinomycin, doxorubicin, vincristine, ribavirin, amiloride and theophylline; hormones such at T_3 and T_4, epinephrine and vasopressin; toxins such as cyanide, carbonylcyanide chlorophenylhydiazone, endotoxins and bacterial lipopolysaccharides; immunological agents such as interleukin-2, epidermal growth factor

35   and monoclonal antibodies; receptor agonists such as isoproterenol, carbachol, prostaglandin E_1 and atropine; and various other types of

cell affecting agents such as phorbol myristate acetate, magnesium chloride, other cells, receptor ligands, viruses, pathogens and pyrogens. This invention also encompasses the measurement of the effects of water immersible cell affecting agents such as particulate

5   matter, greases, and oils on cells. These substances can be delivered to the vicinity of cells by aqueous or non-aqueous fluids.

The present invention includes methods for identifying microbes such as yeast, bacteria, and fungi. In these inventive methods, the bacteria to be identified are trapped or retained within the flow

10 chamber. A predetermined set of solutions and/or suspensions are then sequentially flowed through the micro flow chamber such that they come in contact with the bacteria. Each of these solutions and/or suspensions contains an ingredient that produces a particular response from known bacterium. When the solution and/or suspension

15 containing this ingredient are contacted with bacteria to be identified, the bacteria in the micro flow chamber either produce a characteristic response or produce no response. The response or absence of response is then measured by means of the silicon semiconducting electrode. The response or lack of response to the

20 predetermined set of solutions and/or suspensions may then be compared to the response of known bacteria to the predetermined set of solutions and/or suspensions in order to positively identify the bacteria being tested.

The present invention also includes methods for screening for

25 the activity of a drug. In these inventive methods, living cells that are responsive to the drug to be screened for are trapped or retained within the micro flow chamber. A solution or suspension suspected of containing the drug may then be flowed into intimate contact with these living cells. Upon contact with the living cells,

30 the solution or suspension suspected of containing the drug will produce a response in the living cells or produce no response at all. This effect or lack of effect of the drug on the living cells is then measured by means of the silicon semiconductor electrode. Such drugs may include antibiotics active against microbial cells. The presence

35 or absence of the expected effect may be used as a means for screening solutions or suspensions for the presence of a drug.

- 18 -

The present invention also includes methods for detecting toxic substances. In these inventive methods, living cells that are responsive to the toxic substance being tested are trapped or retained within the micro flow chamber. The solution or suspension suspected of containing the toxic substance is then flowed into intimate contact with the living cells. Any reaction or lack of reaction of the living cells to the solution or suspension is measured, thereby providing a means for detecting the presence of the suspected toxic substance.

The present invention also includes methods for detecting intercellular reactions. In these inventive methods, two sets of living cells are provided within the micro flow chamber. A solution or suspension containing an agent that affects the first set of cells is flowed through the micro flow chamber. This causes the first set of cells to elaborate a second cell affecting agent. The second cell affecting agent in turn causes a response in the second set of living cells. This response is measured by means of the silicon semiconductor electrode. In this way, the intercellular reactions of different sets of living cells may be measured. The first and second sets of cells may be of the same type or may be of different types.

- 19 -

EXAMPLE 1

Keratinocytes are the cells that generate the epidermis. Studies have been done to assess the response of these cells to irritants that might contact the skin in the workplace, in cosmetics, and elsewhere. For example, Shopsis and Eng (Shopsis, C. and Eng, B. (1988) in <u>Alternative Methods In Toxicology</u>, Vol. 6)) determined the concentration of an irritant necessary to inhibit protein synthesis by 50% in a 48-hour incubation of keratinocytes with the irritant. The ranking of a series of detergent irritants by this method agreed well with the rankings obtained by the standard <u>in vivo</u> Draize test of ocular irritancy (Draize, J., Woodard, G. and Clavery, H. (1944) <u>J. Pharmacol. Exp. Ther.</u>, 82:377-390).

Normal human keratinocytes were obtained from Clonetics, Inc. (San Diego, CA) and cultured according to the vendor's instructions. The cells were grown to between 50% and 100% confluency on the conductive indium-tin-oxide surfaces of the glass cover slips used in the cell flow chamber. The irritants tested were benzalkonium chloride (BAC; a cationic detergent), hydrogen peroxide (H_2O_2), 1-butanol (BuOH), ethanol (EtOH), dimethyl sulfoxide (DMSO), propylene glycol, methanol, acetone and sodium dodecyl sulfate.

The cover slip was assembled in the chamber, and flow of medium was initiated. Clonetics provided a low-buffered modification of their Keratinocyte Growth Medium for this purpose; it contained neither bicarbonate nor HEPES buffer.

The flow of medium was controlled by a computer-interfaced syringe pump. Flow was alternately on for 200 sec and off for 200 sec. During the flow-off time, the metabolic rate was determined as the slope of the trace of sensor potential (i.e., pH) vs. time.

Irritants were injected via a 300μl sample injection loop (of the type used in liquid chromatography) at the beginning of a flow-on period.

The flow rates, on the order of 100μml/min, were adjusted so that the irritant was present in the cell chamber during the next flow-off period but had been cleared by the second flow-off period. Typically, samples were injected at 800-sec intervals, allowing two

- 20 -

determinations of metabolic rates for each injection (one with the irritant present, one after it had cleared).

After a stable metabolic rate had been established in the absence of irritants, irritants were injected as described above,
5   working from low to high concentration. Often there were two injections of each concentration. An entire experiment took three to five hours, though a response to each dose of irritant was obtained 400 sec after sample injection. As a control, the metabolic rates of one set of keratinocytes that were administered no irritants were
10 measured over several hours.

The metabolic rate of the control cells remained stable throughout the experiment, with a coefficient of variation of about 6%. FIGURE 11 shows the results for five of the irritants, plotted as metabolic rate vs. the logarithm of irritant concentration.
15 Metabolic rate is expressed as a percent of the value prior to the introduction of irritant. When two successive doses were administered at the same concentration, both sets of data are presented. The large differences in such points at, e.g., $30\mu g/ml$ BAC, represents a real decrease of cellular metabolic rate between
20 the two administrations, not mere scatter in the data. The actual concentration of H_2O_2 may have been less than indicated, due to redox reactions with compounds in the medium as well as disproportionation reactions. For some irritants the metabolic rate rose above the control level for irritant concentrations just below
25 those that were sufficient to inhibit metabolism.

FIGURE 12 is a comparison of measurements taken during the presence of the irritant and after washout. The closed symbols represent rates obtained during the presence of the irritants on the cells; the open symbols correspond to the rates obtained immediately
30 after the irritants were washed out. For one irritant, BAC, no recovery was obtained; in fact, metabolism continued to decrease with each measurement after the higher doses. For EtOH, there was substantial recovery. In a separate test, recover from DMSO but not from ethanol was shown (see FIGURE 13).

35 The irritant strength of these compounds has been given the following classification by _in vivo_ tests such as the Draize test

- 21 -

(see, e.g., Dubin, H. and Ghodgaonkar, R. (1987) In Vitro Toxicology, 1:233-240):

Table I

Irritant	In-Vivo Irritancy	$-\log_{10}(MRD_{50})$
Dimethyl sulfoxide	Mild	0.1
Propylene glycol	Mild	0.5
Methanol	Moderate-mild	0.7
Ethanol	Moderate-mild	0.8
Acetone	Moderate-mild	0.9
n-Butanol	Moderate	1.7
Na dodecyl sulfate	Severe-moderate	3.9
Benzalkonium Cl	Severe	4.1

The ranking of the concentrations necessary to depress metabolism, shown as $-\log_{10}(MRD_{50})$ above, in this example agrees well with that obtained by the in vivo tests.

The inventive method allows flexible control of both the duration and concentration of exposure of the cells to the irritants. Furthermore, it permits one to monitor the effects of these agents on cell metabolism both while the irritants contact the cell and then later, after the irritants have been washed out. Thus, the kinetics of recovery from the insult can be determined along with the main inhibition analysis.

This example demonstrates the feasibility of performing rapid in vitro toxicological assays with the inventive method. Responses to acute exposure have been obtained in a few minutes. Further automation and exploitation of parallelism would bring the time for an entire experiment down to that amount of time. This is a significant advantage in speed and capability over existing in vitro methods.

EXAMPLE 2

Valinomycin is a peptide antibiotic that exerts its effects by inserting itself into cell membranes and rendering them permeable to potassium ions. It is commonly used in research applications where membrane permeability to ions is important. Within no more than a few minutes after being exposed to $100\mu M$ valinomycin, the metabolic

- 22 -

rate of L cells rises significantly; at its peak it may be more than double the initial rate. Following the peak, the rate drops.

FIGURE 14 shows the response to a brief pulse of valinomycin according to the inventive method ($75\mu l$ injected into the medium stream; flow rates about $100\mu L/min$). Similar results are shown in FIGURE 15. In other experiments (graph not shown) the same concentration of valinomycin was administered continuously for 8 minutes and the metabolic activity rose to a peak of approximately 2.4 times the initial value four minutes after the start of exposure. It returned approximately to the initial value within 20 minutes of the removal of the antibiotic. This biphasic response may be an initial expenditure of cellular metabolic energy to attempt to maintain electrolyte homeostasis, followed by the toxic effects of the cells' failure to accomplish this.

EXAMPLE 3

L cells were grown to confluence on the silicon sensor surface of the flow chamber and alternately perfused with normal medium and medium containing 10nM each of the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4). In one experiment when the hormone was first introduced, the metabolic rate increased by 12% and decreased to 9% below the initial level when the hormone was withdrawn. This is in accordance with the expectation that the hormone increases the overall metabolic activity of the cells.

EXAMPLE 4

Phorbol myristate acetate (PMA) is one of the tumor-promoting phorbol esters. Among its effects are an increase in cellular cyclic AMP levels and the deacylation of phospholipids to produce arachidonic acid; it is known to interact with protein kinase C (see, e.g., Daniel, L. (1985) Phosphatidylinositol Turnover in MDCK Cells, in Inositol and Phospoinositides: Metabolism and Regulation, (eds.) Bleasdale, J., Eichberg, J., and Hauser, G. Humana Press). MDCK cells (Madin-Darby Canine Kidney) are an epithelial line that retain many of the functional characteristics of kidney tubule epithelia. One characteristic is that a confluent monolayer of these cells forms a relatively tight seal, both electrically and to the diffusion of ions, separating the aqueous regions on the two sides of the cells.

The sealing property of the MDCK cells implies that when the cells are grown on the surface of the silicon sensor, the signal does not necessarily represent merely the pH of the fluid in the flow chamber. It contains information on the pH of the small aqueous

5 compartment between the cells and the sensor, and it contains information about trans-cellular potentials, which are essentially batteries in series with the electronic circuit of the sensor. In cells that do not form a tight seal, the sub-cellular pH is essentially identical to the flow chamber fluid pH and any trans-

10 cellular potentials are shorted out as far as the sensor is concerned.

FIGURE 16 shows the strong and rapid response that was observed when medium containing 10ng/ml PMA was introduced into the system. The steady-state potential reported by the sensor (with fluid flow

15 on) decreased gradually from the time the sensor was activated until the PMA was introduced. At that time it dropped quickly to a minimum about forty minutes later when the PMA was removed, then increased and finally returned to a value near its initial one by the end of the experiment. This signal is a composite of the trans-cellular

20 potential and the sub-cellular pH (changes in the latter corresponding to 59mV per pH unit).

The introduction of PMA coincided with a sharp rise in the metabolic rate of the cells, more than a doubling at the peak near an hour later. Similar experiments with MDCK cells grown on the cover-

25 slip surface of the chamber give no information about trans-cellular potentials but also indicate an increase in metabolic rate upon the addition of PMA (data not shown).

The metabolic rates determined from cells grown on the sensor surface are more sensitive to the treatment of the cells and

30 generally less stable than those from cells grown on the cover slips. This is understandable in terms of the small volume of the sub-cellular aqueous compartment compared to the volume of the chamber; small changes in transport of carbon dioxide and protons across cell membranes can cause larger changes in pH in smaller volumes.

35 The effects of PMA on steady-state potential are particularly interesting in light of the fact that PMA is known to disrupt the

- 24 -

tight junctions responsible for the resistive sealing between these cells. One would expect, therefore, PMA to short out the effects of trans-cellular potentials and to make the composition of the sub-cellular space more like that of the general flow chamber fluid.

EXAMPLE 5

Cyanide (CN^-) is a anion that binds to the oxygen combining site of cytochrome oxidase and inhibits electron transport (thereby inhibiting cell respiration). Cells become glycolytic after being exposed to CN^-. L Cells (a mouse fibroblast cell line) were grown to confluence on the silicon sensor surface of the flow chamber and exposed to an injection of 100 μM sodium cyanide. Within minutes, the metabolic rate increased 39% above the rate when the compound was introduced, and then the rate began to gradually decrease. At this point, the cells were rounded up and beginning to die.

EXAMPLE 6

BHK cells (a fibroblast line from baby hamster kidneys) were grown on the silicon sensor surface of the flow chamber and alternately perfused with normal medium and medium containing 0.6 or 0.06 E.U. of bacterial lipopolysaccharide endotoxin. As shown in FIGURE 17, the introduction of 0.06 E.U. of endotoxin (with 0.001% BSA as a protein carrier) resulted in a 31 to 36% increase in metabolic rate over the rate obtained when the cells were in the presence of medium only (control value). The metabolic rate remained unchanged (with respect to the rate observed when cells were in the presence of medium only) when medium with 0.001% BSA (bovine serum albumin) or medium with 0.01% BSA plus 0.6 E.U. of endotoxin was introduced. This result may reflect a biphasic response of BHK cells to endotoxin. This is frequently seen for receptor mediated stimulation of cells. A low dose of stimulant gives a response whereas a very high dose can give no response or an opposite response compared to a low dose.

EXAMPLE 7

Amiloride is a diuretic drug that has been used extensively as an inhibitor of the Na^+ channel of transporting epithelial cells present in the kidney and the toad bladder. Taub et al. demonstrated that the MDCK cell line possesses an amiloride-sensitive Na^+ channel

- 25 -

characteristic of the cells present in the distal tubule of the
kidney (<u>J. Cellular Physiology</u>, 106:191-199 (1981)). When MDCK cells
were grown on the cover slip portion of the flow chamber, the
introduction of 1.5 mM amiloride resulted in a gradual increase of

5   metabolic rate up to 16% above the rate observed for medium only.
Once exposure to the drug was ceased, the rate returned to the
original control value and then gradually continued to decline.

When MDCK cells were grown on the silicon sensor surface of the
flow chamber, the introduction of 1.5 mM amiloride resulted in an

10   immediate 20% decrease in apparent metabolic rate with respect to the
rate observed in the presence of medium only.

Interpretation of the data collected from MDCK cells was made
difficult by the fact that as cell respiration made the medium more
acidic (after medium flow was stopped), the cells responded to the

15   change in pH thereby resulting in a change in the original metabolic
rate, to which the cells could also respond. This complicated cell
response resulted in a multiphasic curve when medium flow was stopped
for a period of time. In the case where the cells were grown on the
silicon sensor surface, this phenomenon was particularly dramatic.

20   <u>EXAMPLE 8</u>

Theophylline (a diuretic, cardiac stimulant, and smooth-muscle
relaxant) has been shown to inhibit phosphodiesterase, which results
in an increase in the amount of cAMP in the cell. When MDCK cells
were grown on the silicon sensor surface, the introduction of 10 mM

25   theophylline resulted in a 47% decrease in the metabolic rate with
respect to the control value. After the theophylline containing
medium was removed, the metabolic rate returned to the control value
within ten minutes. While the therapeutic level of theophylline
($111 \mu M$) introduced to MDCK cells grown on the silicon sensor surface

30   resulted in an approximate 10% decrease in metabolic rate, a 28%
increase in rate with respect to the control values was observed when
theophylline was removed from the cell environment.

MDCK cells grown on a cover slip demonstrated a similar decrease
in the metabolic rate (59%) when the cells were exposed to 10 mM

35   theophylline, although a gradual increase in rate was observed (up to
only 48% below the control value). Once the theophylline was removed

- 26 -

from the cell environment, the metabolic rate increased 30% over the control value.

EXAMPLE 9

MDCK cells are known to have epinephrine receptors on their
5   basolateral surface (the surface of the cells which attaches to the substrate on which they are growing).   Therefore, when MDCK cells are grown on the silicon sensor surface, the epinephrine receptors are oriented on the sensor side of the cellular monolayer.   When medium containing $3\mu M$ epinephrine was flowed over the cell monolayer,
10   the epinephrine had to diffuse across the cell layer to reach the receptor.   The primary introduction of medium plus epinephrine did not significantly alter the metabolic rate with respect to the control rate.   However, as shown in FIGURE 18, additional exposure to medium plus epinephrine resulted in a 20% increase in metabolic rate.
15   The metabolic rate remained 12% higher than the control rate after the epinephrine containing medium was removed from the cell environment.   An additional 7% increase in metabolic rate was attained when the cells were once again exposed to the epinephrine containing medium (i.e. a 20% increase in metabolic rate over the
20   control rate).

EXAMPLE 10

Another method to immobilize cells is to encapsulate them in an agarose gel.   MDCK cells in a confluent T-75 flask were trypsinized in order to remove them from their growth container.   The cells were
25   centrifuged at 2000 RPM for 5 minutes and the supernatant was discarded.   The cells were resuspended in 5 mL of medium and centrifuged for 5 minutes at 3000 RPM.   The supernatant was again discarded, and the cells were resuspended in $500\mu L$ of 1.2% sea plaque agarose from FMC (in medium).   The cells were centrifuged for 3
30   minutes at 3000 RPM to pellet the cells.   A $2\mu L$ aliquot was taken directly from the cell pellet and spread over an approximate area of 40 mm^2 on the silicon sensor surface (in a partially assembled chamber).   The chamber was refrigerated for 15 minutes in order to allow the agarose to gel.   The assembly of the flow chamber was then
35   completed and the cells were alternately perfused with medium only, medium plus $208\mu M$ epinephrine, or medium plus $3\mu M$ epinephrine.   The

- 27 -

presence of 208μM epinephrine resulted in a 3% decrease in the
metabolic rate with respect to the rate observed for medium only.
However, as shown in FIGURE 19, the removal of the epinephrine from
the cell environment resulted in a 14% increase in metabolic rate
5   over the control rate.   Similarly, the introduction of 3μM
epinephrine to the cells resulted in a 4.5% decrease in metabolic
rate with respect to the rate observed immediately after the higher
epinephrine concentration was removed.   When the 3μM epinephrine was
removed from the cell environment, the metabolic rate increased 17%
10   over the rate observed prior to its introduction.

EXAMPLE 11

CTLL cells (a cytotoxic T-Cell line from mice) are a nonadherent
cell line that requires the presence of Interleukin-2 (IL-2; an
immune modulator) to remain viable.   Two mL of cells were removed
15   from a confluent T-25 flask of CTLL cells and centrifuged to pellet
the cells.   The supernatant was discarded and the cells were
resuspended in 300μL of a 2% agarose solution (in medium).   The cells
were again centrifuged to a pellet, and a 2μL aliquot was removed
from the cell pellet and spread over an approximate 40 mm^2 circle on
20   a cover slip that was housed in a humidified chamber.   The cover slip
chamber was then refrigerated for 20 minutes to allow the agarose to
gel.   The cover slip was removed from the humidified chamber and
placed in a flow chamber.   Medium containing 20 units/mL of IL-2 or
medium only was alternately flowed across the cells.   When the medium
25   containing IL-2 was removed, as shown in FIGURE 20, the metabolic
rate began to decline with respect to the control rate.   The rate
continued to decline until medium containing IL-2 was reintroduced to
the system, at which time the rate began to increase (although the
rate did not return to the control rate value).   At this time, the
30   cells were beginning to detach from the agarose gel.   As expected,
as the cell density decreased, so did the rate of acidification of
the chamber.

EXAMPLE 12

L cells were grown to confluence on the silicon sensor surface
35   of the flow chamber and alternately perfused with normal medium and
medium containing 0.6 E.U. of bacterial lipopolysaccharide endotoxin.

- 28 -

In one experiment the introduction of endotoxin increased the initial metabolic rate 20% above the rate observed for medium only, and a second exposure of the cells to endotoxin increased the rate 15% above the rate observed for medium only. In both cases, the rate

5   returned to the initial value when the endotoxin containing medium was removed (within a few minutes).

EXAMPLE 13

Oxytocin is the principal uterus-contracting and lactation-stimulating hormone of the posterior pituitary gland. The

10   introduction of $1\mu M$ oxytocin to MDCK cells grown on a cover slip did not result in any immediate change in the metabolic rate with respect to the control value, although the metabolic rate did gradually decrease (up to 10%). The removal of the oxytocin from the cell environment did not immediately return the cell metabolic rate to its

15   control value, although within 10 minutes 100% recovery was attained. This was a control experiment since MDCK cell do not have oxytocin receptors, and oxytocin is a nonapeptide which differs from vasopressin by only two amino acids. MDCK cells do have vasopressin receptors. This was not necessarily the best control experiment

20   because, as shown below, only cells grown on the silicon sensor surface demonstrated a response to vasopressin.

EXAMPLE 14

The introduction of $0.1\mu M$ vasopressin (antidiuretic hormone) to MDCK cells grown in the silicon sensor surface resulted in a 14%

25   increase in metabolic rate over the control values. The addition of $1\mu M$ vasopressin immediately after the $0.1\mu M$ vasopressin resulted in another increase in the metabolic rate (19% over the rate attained in the presence of $0.1 \mu M$ vasopressin, which is a 42% increase over the control rate value).

30   When $1\mu M$ vasopressin was introduced to MDCK cells grown on a cover slip, no increase in metabolic rate was observed.

EXAMPLE 15

The addition of 10 mM MgCl to the control medium resulted in a 40-46% increase in metabolic rate with respect to the metabolic rate

35   observed when the MDCK cells were exposed to medium only.

- 29 -

EXAMPLE 16

A silicon semiconductor electrode with a nylon mesh adhesively attached to its upper surface, as described above, was prepared. CTLL cells were flowed into the chamber at a flow rate of approximately 10μL/min. At this flow rate some of the cells settled by gravity into the holes in the nylon mesh. The flow rate was then increased to approximately 100μL/min and the cells remained in the holes. Cells could be visualized in the microscope. The laser beam was directed to three different regions where cells were present and one region where no cells were present. At each site the change in pH with time was measured with the medium flow on and off. The slope of the pH change in μV/sec with the flow off minus the same change with flow on was as follows:

Table II

(Flow Off-Flow On)

Measurement	μV/Sec	S.D.	Cells
1	-9.7	.92	present
2	-6.2	.64	present
3	-4.4	.85	present
4	+1.7	.71	absent

EXAMPLE 17

This experiment was designed to demonstrate the possibility of determining partial or complete identification of bacteria on the basis of their ability to change the pH of their immediate environment in the presence of several potential carbon source compounds supplied one at a time in a serial manner to a single "catch" of bacteria. While this method of serial delivery of selective substrates has certain potential advantages, parallel delivery of selective substrates to separate catches of bacteria could also be useful under certain circumstances. Furthermore, if bacteria are used for screening for antibiotic susceptibility or antibiotic activity, parallel delivery to separate catches may well be more appropriate because of potential problems of intereactions of drugs.

- 30 -

The bacteria, E. coli and Proteus mirabilis, were prepared as suspensions of about 5 x 10^8 cells/mL in a growth medium and then diluted into a sterile test medium containing glucose (Bactopeptone, 2g/L; NaCl, 5 g/L; K_2HPO_4, 1.7mM; glucose, 1 g/L; pH 7.3). With the controlling electrode piston in the raised position, as disclosed in pending U.S. Patent Application, Serial No. 876,925, filed June 20, 1986, one mL of bacterial suspension containing a known number of bacteria was flowed through the device such that the cells were retained adjacent to the silicon sensor surface by a Nucleopore membrane situated parallel to the surface. The cells were pulsed with the same glucose containing test medium, the piston was then lowered to decrease the volume surrounding the cells, and the change in pH due to bacterial metabolism was monitored. The piston was then raised and a second test medium containing urea in place of glucose was flowed through. After lowering the piston the pH change was again monitored. This procedure was repeated with medium containing pyruvate as a carbon source.

FIGURE 21 shows that E. coli respond by decreasing the pH in the presence of all three media (each medium was tested three times in succession before testing the next medium). FIGURE 22 shows that P. mirabilis decreased the pH in the presence of glucose and pyruvate, but increases the pH in the presence of urea. FIGURE 23 shows that the rate of pH change in glucose medium is dependent on the number of bacteria trapped in the device.

FIGURE 24 shows E. Coli in a system that differs from that used for the experiments shown in FIGURES 21 and 22 in that the cells were retained by a membrane, the plane of which was parallel to the flow of the medium rather than perpendicular.

In FIGURE 24, the E. Coli demonstrates an increase in pH change amplihide with time consistent with an increase in the number of bacteria with time. In addition to this apparent demonstration of bacterial replication in the flow cell, FIGURE 24 shows that E. Coli do not respond to an antibiotic that has low activity against gram negative bacteria (penicillin added at point A) but do respond to an antibiotic active against gram negative bacteria (polymyxin added at point B).

- 31 -

EXAMPLE 18

CTLL cells were trapped in the flow chamber in rectangular wells. Referring to FIGURE 25, an array 1 of $1000\mu m$ long, $50\mu m$ wide slots, with $50\mu m$ separation between slots, was cut into a $50\mu m$ thick piece of stainless steel foil 2 with an eximer laser. This was mounted in the $100\mu m$ deep flow chamber under pieces of $50\mu m$ thick plastic 3 arranged to press the foil against the silicon semiconductor electrode surface when the cover slip was mounted. This left a channel about 1mm wide and $50\mu m$ deep above the slots, thereby forming wells.

CTLL cells were settled into the wells at a linear flow velocity of the medium in the channel of no more than about $15\mu m/sec$. Alternatively, the wells could be loaded by flowing the medium faster than $15\mu m/sec$. until the cells were suspended above the wells, and then stopping the flow entirely for approximately 10 seconds while the cells sedimented into the wells. This flow/stop procedure was repeated until sufficient cells were trapped.

The cells remained in the wells for flow rates below approximately $100\mu m/sec$, above which many were flushed out. Alternatively, momentary inversion of the chamber caused the cells to sediment into the flow stream and be flushed away at lower flow rates.

The thin film of liquid between the foil and the silicon electrode, plus possible corrosion of the stainless steel, makes the baseline potential somewhat unstable. This problem could be corrected by micro-machining the wells directly into the silicon semiconductor electrode.

Metabolic rates of -30 to $-60\mu V/sec$ were obtained from CTLL cells. This is close to the range observed with adherent cells. Experiments with IL-2 similar to those in Example 11 suggested that after several hours without IL-2 the cells' metabolic rate had decreased to about 20-30% of its control value; after several hours with IL-2 (20U/ml) other cells' rate was over 50% of the control value. This is an indication that the presence of IL-2 can be detected on the time scale of a few hours. Optimization of the apparatus may shorten that time considerably.

- 32 -

EXAMPLE 19

Three cell lines, provided by Dr. Craig Venter at the NIH, have enabled us to detect the functional interaction of agonists and antagonists with specific receptors. The basic cell line, called 5 B82, is a derivative of the L cell line (mouse fibroblast line) and is thought to contain only one natural receptor (the prostaglandin E_1 (PGE_1) receptor). These cells function only as a control for our experiments.

We have also obtained the cell line with two different 10 transfections. The line designated M1C-2 is the B82 line into which a rat M_1 muscarinic receptor has been transfected. The line designated 821 is the B82 line into which a human B_2-adrenergic receptor has been transfected. Venter and coworkers have determined that the transfections are functional, in the sense that agonists 15 stimulate the appropriate second-messenger systems (phosphoinositol for M1C-2, cyclic AMP for 821). The characterization of these and similar cells can be found in Fraser, C., Chung, F.-Z., and Venter, J.C. (1987) Continuous high density expression of human B_2-adrenergic receptors in a mouse cell line previously lacking B-20 receptors J. Biol. Chem. 262:1483-1486; Mei, L., Lai, J., Roeske, W., Fraser, C., Venter, J.C., and Yamamura, H. (1989) Pharmacological characterization of the M_1 muscarinic receptors expressed in murine fibroblast B82 cells. J. Pharmacol. Exp. Therapeutics 248:661-670.

Each of the three cell lines is grown in F-12 DME medium 25 supplemented with 10% fetal bovine serum. Geneticin (50 μg/ml) is always included in the 821 cells' medium to prevent a reversion of the transfection. The cells of interest are plated on indium-tin oxide coated cover slips and allowed to grow to 60-90% confluence. Sixteen to twenty-four hours prior to the experiment, the growth 30 medium is removed and replaced with medium containing no fetal bovine serum. In the case of the M1C-2 cells, 1 μM dexamethasone is added with the serum-free medium and in subsequent steps to induce the transcription of the M_1 gene which is under the constitutive control of the dexamethasone-inducible MMTV promoter.

35 After the cells have been serum starved, the cover slips are loaded into the flow chamber with the cells facing the silicon

- 33 -

sensor. Low-buffered medium (F-12 DME without sodium bicarbonate or HEPES buffer added) is flowed through the cell chamber at ~100 μl/min. The cell chamber is kept at 37μ with a flow cycle of 300 sec on and 200 sec off. The cells are allowed to equilibrate in control
5   medium for two to three hours before the introduction of the agonist. Time zero on the following graphs indicate the time at which agonist and/or antagonist was introduced to the cell chamber.

FIGURE 26 is an example of an isoproterenol dose-response curve using 821 cells. The cells exposed to the control medium remained
10 relatively constant, whereas isoproterenol caused a rapid increase in the metabolic rate, peaking within the first 30 minutes. A dose-response relationship was observed.

FIGURE 27 shows the effect of carbachol (an M_1 agonist), PGE_1, and isoproterenol on M1C-2 cells, which contain both the M_1
15 muscarinic and PGE_1 receptors. Carbachol caused an 80% increase in metabolic rate, which peaked in approximately 50 seconds and dropped back to a steady state level of approximately a 40% increase in metabolic rate in 200 seconds. Stimulation of M1C-2 cells with PGE_1 resulted in an 80% increase in metabolic rate that peaked in
20 approximately 1000 seconds and returned to base line metabolic rates in 4000 seconds. The difference in the peak timing can be attributed to the fact that the binding of PGE_1 to the PGE_1 receptor affects a different second-messenger pathway (cyclic AMP) than does the binding of carbachol to the muscarinic receptor. FIGURE 27 also demonstrates
25 the lack of B_2-adrenergic receptors in M1C-2 cells, since the introduction of isoproterenol had no effect.

FIGURE 28 demonstrates agonist-antagonist interactions with the muscarinic receptor in M1C-2 cells. The introduction of 10 mM carbachol (a muscarinic agonist) to M1C-2 cells resulted in a 75%
30 increase in metabolic rate with respect to baseline metabolic rates. Atropine, a potent muscarinic antagonist, blocked the response of these cells to carbachol. Atropine alone had no significant effect on the cells.

FIGURES 29 and 30 show results obtained with a protocol modified
35 slightly to increase the temporal resolution of the rapid response of the M1C-2 cells to carbachol. Two chambers (one experimental and one

- 34 -

control) were assembled with cells and equilibrated as described above,

During an off period, the flow rate was changed from 100 μl/min to 200 μl/min. When the flow resumed, an injection valve was switched to introduce carbachol containing medium into the cell chamber. The flow was stopped after 45 seconds and the acidification of the medium was followed (FIGURE 29). The time derivative of the sensor potential (equivalent to pH) was calculated using a 5 point quadratic Savitsky-Golay (1964) algorithm and is shown in FIGURE 30. (See Savitsky, A., and Golay, M. (1964) Smoothing and differentiation of data by simplified least squares procedures. Analytical Chem 36:1627-1639.)

Thus, the rate of acidification can be followed with an improved resolution of ˜2 seconds over the period in which the flow is off. The duration of this experiment is set by the length of time it takes the cells to perturb the pH and metabolite concentrations of the stagnant medium significantly. At this point, flow must be restored and data collectionhalted.

The response peak occurred 50-60 seconds after the cessation of medium flow. It should be noted that because a 5 point Savitsky-Golay algorithm was used in the calculations, the first few points are not valid; they include data points during the time of medium flow.

The control cells in this example is the B-82 cell line, a murine fibroblast cell line. The B-82 cells contain a prostaglandin E_1 receptor. They do not contain either muscarinic or adrenergic receptors. B-82 cells have been transfected with the human $beta_2$-adrenergic receptors (821 cells) and with rat M_1 muscarinic cholinergic receptors (M1C-2 cells). The cell lines were provided by Dr. J. C. Venter. All the above mentioned cell lines are grown in F-12 DME with 10% Fetal Bovine Serum (FBS). The transfected 821 cell growth medium contained 50 μg/ml geneticin to prevent reversion of the transfection. Geneticin is included in the low buffered experimental medium also. Each of the cell lines are plated on indium-tin oxide covered cover slips and allowed to grow to 85-100% confluence. Approximately twenty-four hours prior to the experiment,

the growth medium is removed and replaced with serum depleted medium. In the case of the M1C-2 cells, 1μM dexamethasone is added to the serum depleted medium.

The cover slips are then loaded into the flow chamber with the
5   cells facing the silicon sensor.   Low buffered medium is flowed through the chamber (kept at 37°).   821 cells are exposed to medium with 50 μg/ml geneticin. M1C-2 cells are exposed to medium with 1 μM dexamethasone.   B82 cells are exposed to medium with 0.5% ethanol (because ethanol is needed to dissolve PGE_1).   After the cells are
10   allowed to equilibrate for approximately 2 hours, the receptor agonist of interest is introduced (100 μM PGE_1 is introduced to B82 cells, 10 μM isoproterenol to 821 cells, and 3.12 μM carbachol to M1C-2 cells).   Time 0 on these figures indicates where the valves are switched to introduce the agonists.   It should be noted that there is
15   a slight delay before the agonist actually enters the flow chamber.

EXAMPLE 20

The infection of cells in tissue culture by vesicular stomatitis virus (VSV) results in the alteration of cellular metabolism as a consequence of shut-down of protein and RNA synthesis.   The result of
20   viral infection and replication is cytolysis of host cells, which is preceded by cytopathic effects, i.e., cell swelling and rounding. These morphological consequences of infection can be used to detect virus replication in cell culture.   In theory, modification of host cell metabolism could also be used to detect and monitor viral
25   replication.   In the present example, a silicon-based biosensor, which measures production of acidic metabolites, has been used to monitor metabolism of mouse L cells during VSV infection.   Infected and uninfected cells are held in a thin (100μm) flow chamber which enables sensitive and quantitative measurement by the biosensor of
30   medium acidification during stopped flow conditions.   Between metabolic measurements, fresh medium is perfused through the flow chamber to maintain the cells under optimal growth conditions.   Using the biosensor system, a depression in L cell metabolic rate was detected between 2-4 hours post VSV infection and a precipitous drop
35   in rate occurred later in the cycle, 8-14 hours post infection (See FIGURES 31 and 32).   The extent and time at which host cell

- 36 -

metabolism decreased were found to be a function of multiplicity of infection (See FIGURES 33 and 34). The specificity of the VSV effect on host cell metabolism was confirmed by sensitivity to UV irradiation of viral inoculum (See FIGURE 35), by neutralization with mono-

5   clonal anti-VSV antibody (See FIGURE 36), and by inhibition when ribavirin was included in the medium (See FIGURES 37A and 37B). We have also demonstrated the protection by mouse Interferon of the host L cells from VSV infection, using the biosensor (See FIGURES 38 and 39).

10   FIGURES 40, 41A, 41B and 41C show additional data generated by the infection of L cells by VSV, Influenza A (WSN strain) and HSV-1. Influenza A is a minus-strand RNA virus that causes influenza in man. HSV-1 is a DNA virus that cause herpes infection in man. Acyclovir may be used to inhibit HSV-1 and amantadine may be used to inhibit

15   influenza A.

EXAMPLE 21

For many types of mammalian cells, a confluent monolayer (equivalent to about 10^7 cells/mL) commonly produces acidification rates within a factor of two of $100\mu V/s$. At 59 mV per pH and medium

20   buffer capacity of 1mM, this corresponds to a calculated net rate of H+ production of $^-1\cdot10^8$/s for a typical cell. The metabolic sources of these protons depend on the nutrients in the culture medium and the metabolic pathways that are active in the cells. Some insight into this can be gained using auxiliary biochemical measurements and

25   specific metabolic poisons.

Oxygen consumption was measured in a separate perfusion apparatus equipped with a Clark oxygen electrode. The removal of oxygen from the media by a monolayer of the murine fibroblastic L cell line was measured in the output stream under conditions of constant flow.

30   Lactate production was also measured, using a spectrophotometric enzymatic assay based on the reduction of NAD+. When culture medium was used as the bathing medium, the rates of oxygen consumption (approximately corresponding to CO_2 production) and lactate production per cell were found to be roughly equal to $3.1(\pm0.3)\cdot10^7$/s.

35   Similar results were obtained when the cells were tested in a balanced salt solution with 5mM glucose as the only carbon source.

SUBSTITUTE SHEET

- 37 -

The metabolic poison carbonylcyanide chlorophenylhydrazone (CCCP) is a respiratory uncoupler that has been shown to simulate mitochondrial oxygen uptake. FIGURE 42 shows the effect of 20 μM CCCP on the non-adherent murine macrophage-like line P388D1 trapped
5   in etched wells. The stimulation is prompt, faster than the resolution of the apparatus. It is reversible, with a recovery time of about 20 min. after the removal of the uncoupler. Dose-response experiments show a maximum stimulation of the acidification rate near 2-5 μM CCCP. Presumably the mechanism is a combination of increased
10 CO_2 production and the stimulation of glycolysis to maintain homeostasis.

EXAMPLE 22

Normal human keratinocytes require epidermal growth factor (EGF) for proliferation without differentiation. Keratinocytes (from
15 Clonetics, Inc.) were cultured in the absence of EGF for a period of 24 hours prior to the experiment. For the experiment, the keratinocytes were assembled into four flow chambers and the alternating stop-flow sequence was initiated with medium containing no EGF. At the time indicated by the arrow in FIGURE 43, the
20 syringes containing medium were changed on all four flow chambers. The second medium contained 5 ng/ml mouse EGF, or 1.25 μg/ml of a neutralizing rabbit anti-mouse-EGF polyclonal IgC, or both. A control with no EGF or IgC was also run. The addition of EGF led to a metabolic burst by the keratinocytes that was neutralized by
25 inclusion of the anti-EGF antibody. Addition of the anti-EGF antibody alone had no effect.

These experiments, the results of which are shown in FIGURE 43, demonstrate that the microphysiometer is capable of detecting some ligand-receptor interactions functionally in living cells.

30 In a separate experiment, the growth medium for the normal human epidermal keratinocytes contained 10 ng/mL murine EGF, a substance found to be mitogenic for keratinocytes. FIGURE 44 shows that, after EGF starvation for 24 hr, a rise in metabolic rate is evident within 5 min. after the introduction of 50 ng/mL EGF to keratinocytes in the
35 silicon microphysiometer. At the peak of the response, in ~15 min, the metabolic rate has nearly doubled; in another 15 minutes it has

- 38 -

decayed to a steadily rising baseline. A parallel control experiment with a sham change of EGF-free medium, also depicted in FIGURE 44, displays none of this biphasic behavior. It does show the same rising baseline, which probably indicates some combination of cell
5   proliferation and adaptation to the environmental conditions in the flow chamber.

Stimulation of acidification rate was observable down to 0.1 ng/mL EGF. In a control experiment, the stimulation was abolished by a monoclonal antibody to EGF that is known to block receptor binding.
10   These experiments demonstrate the ability of the biosensor to detect a cellular response to ligand-receptor binding on a time scale of minutes.

The effects of EGF on keratinocytes closely parallels that previously reported for mouse and human fibroblasts, where
15   stimulation with EGF has been shown to lead to a rapid increase in glycolytic rate. The enhancement in rate has been attributed to increased 6-phosphofructo-1-kinase activity as a result of increased concentrations of fructose-2,6-biphosphate, a potent stimulator of the enzyme. Fructose-2,6-biphosphate has also been shown to play a
20   role in the stimulation of glycolysis in fibroblasts by serum, insulin, and phorbol esters. Modulation of glycolytic rates in response to hormonal activation has been reported in other cell types, including muscle and liver cells, thus lending support to the measurement of metabolic activity as a means of detecting hormonal
25   stimulation.

EXAMPLE 23

The cytotoxic effects of chemotherapeutic drugs on tumor cell lines are commonly determined by assays that detect proliferation. Using a previously characterized cellular system, we have performed
30   cytotoxicity assays in the silicon microphysiometer. MES-SA is a human uterine sarcoma line that is sensitive to doxorubicin and vincristine. The Dx5 line, derived by selection with doxorubicin from MES-SA, is resistant to doxorubicin and vincristine by virtue of enhanced activity of the P-glycoprotein transporter, which confers
35   multidrug resistance.

- 39 -

Experiments using the silicon microphysiometer reproduce these results.   At   therapeutic   doses   of   doxorubicin   (1 μM),   the differential   cytotoxicity   in   the   two   lines   is   maintained.   As depicted in FIGURE 45, decreased acidification rates are evident at
5   15 hours MES-SA but not by 50 hours for Dx5. At high (100 μM) doses of the drugs similar but more rapid results occur: cytotoxicity of doxorubicin is manifest by 0.5 hour for MES-SA and 2.5 hours for Dx5. The results for 100 μM vincristine, shown in FIGURE 46, are 5 and 10 hours, respectively.

10   This system requires relatively few cells and no extended cell growth.   These   factors,   plus   the   ability   of   making   repeated measurements   on   the   same   set   of   cells,   suggest   additional applications in cytotoxicity testing.

The present invention has been described in terms of certain
15   preferred embodiments.   Other embodiments not specifically described herein   may   nevertheless   fall   within   the   spirit   or   scope   of   the present invention or the following claims.

- 40 -

WE HEREBY CLAIM AS OUR INVENTION:

    1.   A method for detecting an effect of a cell affecting agent on living cells comprising:

    (a) providing living cells retained in a micro flow chamber adapted for continuous or intermittent flow of solutions or suspensions containing the cell affecting agent in intimate contact with the cells such that the amount of the cell affecting agent in contact with the cells can be controlled;

    (b) flowing a solution or suspension containing the cell affecting agent such that it comes into intimate contact with the living cells thereby producing a cell mediated extracellular effect, said cell mediated extracellular effect being selected from the group consisting of pH, redox potential, cell surface potential and trans-cellular potential; and

    (c) measuring the effect of the cell affecting agent by a means for detecting the effect operatively associated with the micro flow chamber.

    2.   The method of Claim 1 wherein the means for detecting the effect of the cell affecting agent comprises a silicon semiconductor electrode in contact with or in the immediate vicinity of the living cells.

    3.   The method of Claim 2 wherein the living cells are adhered to the surface of the silicon semiconductor electrode.

    4.   The method of Claim 2 wherein the living cells are retained in wells in the vicinity of the silicon semiconductor electrode.

    5.   The method of Claim 2 wherein the living cells are retained in a compartment in the vicinity of the silicon semiconductor electrode by means of a membrane permeable to the cell affecting agent.

    6.   The method of Claim 2 wherein the living cells are adhered to a membrane or microcarrier in the vicinity of the silicon semiconductor electrode.

    7.   The method of Claim 1 wherein the living cells are adhered to a surface of the micro flow chamber.

8.   The method of Claim 1 wherein the living cells are adhered to a membrane or microcarrier within the micro flow chamber.

9.   The method of Claim 1 wherein the living cells are retained in wells in the micro flow chamber.

10.   The method of Claim 7 wherein the cells are adhered by spontaneous or natural adhesion.

11.   The method of Claim 7 wherein the cells are removably adhered by means of a biologically compatible adhering agent.

12.   The method of Claim 11 wherein the adhering agent is agarose.

13.   The method of Claim 1 wherein the cells are retained in a compartment in the micro flow chamber by means of a membrane permeable to the cell affecting agent.

14.   The method of Claim 1 wherein the solution or suspension is partially degassed prior to coming into intimate contact with the cells.

15.   The method of Claim 2 wherein the effect of the cell affecting agent is measured at a plurality of sites on the silicon semiconductor electrode.

16.   The method of Claim 3 wherein the cells are adhered to the surface of the silicon semiconductor electrode by spontaneous or natural adhesion.

17.   The method of Claim 3 wherein the living cells are removably adhered to the surface of the silicon semiconductor electrode by means of a biologically compatible adhering agent.

18.   The method of Claim 17 wherein the adhering agent is agarose.

19.   The method of Claim 1 wherein the solution or suspension is continuously flowed in intimate contact with the cells.

20.   The method of Claim 19 wherein the pH of the solution or suspension is measured at a plurality of sites in the micro flow chamber.

21.   The method of Claim 1 wherein the solution or suspension is intermittently flowed in intimate contact with the cells.

22.   The method of Claim 21 wherein the pH of the solution or suspension is measured when the flow is stopped.

- 42 -

23. The method of Claim 1 wherein the effect measured is pH.

24. The method of Claim 1 wherein the effect measured is redox potential.

25. The method of Claim 1 wherein the cell affecting agent is a drug, hormone, virus, toxin or immunological agent.

26. A method for microbial identification comprising:

(a) providing a micro flow chamber adapted for continuous or intermittent flow of solutions or suspensions through the micro flow chamber wherein the micro flow chamber has a means for trapping microbes to be identified on or in the micro flow chamber;

(b) sequentially flowing a predetermined set of solutions and/or suspensions through the micro flow chamber where each solution or suspension contains one or more ingredients that provide a microbe response characteristic of the microbe being tested when contacted with the microbe to be identified;

(c) measuring the microbe response to each solution or suspension by a means for detecting the response operatively associated with the micro flow chamber; and

(d) comparing the measured microbe response to the predetermined set of solutions and/or suspensions to the response of known bacteria thereby identifying the bacteria to be identified.

27. A method for screening for the presence or activity of a drug comprising:

(a) providing a micro flow chamber, said micro flow chamber being adapted for continuous or intermittent flow of solutions or suspensions containing a drug to be tested through the micro flow chamber;

(b) further providing living cells responsive to the

drug in the micro flow chamber in contact with or
in the micro flow chamber;

(c)   contacting the living cells with the drug to be
tested by flowing a solution or suspension of the
drug to be tested through the micro flow chamber;
and

(d)   measuring an effect of the drug to be tested on
the living cells by a means for detecting the
effect operatively associated with the micro flow
chamber.

28.   The method of Claim 27 wherein the drug is an antibiotic.

29.   The method of Claim 27 wherein the drug is an antiviral
agent and the living cells are infected with a virus.

30.   A method for detecting a toxic substance comprising:

(a)   providing a micro flow chamber, the micro flow
chamber being adapted for continuous or
intermittent flow of a solution or suspension
suspected of containing a toxic substance;

(b)   further providing living cells responsive to the
toxic substance being tested in the micro flow
chamber in contact with or in the micro flow
chamber;

(c)   contacting the living cells with the solution or
suspension suspected of containing the toxic substance
by flowing the solution or suspension through the
micro flow chamber; and

(d)   measuring an effect of the toxic substance on the
living cells by a means for detecting the effect
operatively associated with the micro flow
chamber.

31.   A method for detecting intercellular reactions comprising:

(a)   providing a micro flow chamber adapted for
continuous or intermittent flow of solutions or
suspensions through the micro flow chamber;

(b)   further providing a first and second set of
living cells in the micro flow chamber wherein at

- 44 -

least the second set of living cells is in contact with or in the micro flow chamber;

(c) contacting the first set of living cells with a first cell affecting agent by flowing a solution or suspension containing the first cell affecting agent through the micro flow chamber, thereby causing the first set of living cells to elaborate a second cell affecting agent; and

(d) measuring an effect of the second cell affecting agent on the second set of living cells by a means for detecting the effect operatively associated with the micro flow chamber.

32. The method of Claim 31 wherein the first and second sets of living cells are the same type of living cells.

33. The method of Claim 31 wherein the first and second sets of living cells are different types of living cells.

34. An apparatus for detecting an effect of a cell affecting agent on living cells comprising:

(a) a micro flow chamber having a plurality of internal surfaces, one of the internal surfaces being a floor, the micro flow chamber being adapted to retain living cells therein, the micro flow chamber being further adapted for continuous or intermittent flow of solutions or suspensions containing the cell affecting agent in intimate contact with the cells such that the amount of the cell affecting agent in contact with the cells can be controlled;

(b) means for retaining living cells in the micro flow chamber;

(c) means for flowing a solution or suspension containing the cell affecting agent such that it comes into intimate contact with the living cells retained in the micro flow chamber thereby producing a cell mediated extracellular effect, said cell mediated extracellular effect being selected from the group consisting of pH,

- 45 -

redox potential, cell surface potential and trans-
cellular potential;

and

(d)   means for measuring the effect of the cell affecting
      agent, the means being operatively associated with the
      micro flow chamber.

35.   The apparatus of Claim 34 wherein the means for retaining
living cells in the micro flow chamber comprises a plurality of wells
in the floor of the chamber.

36.   The apparatus of Claim 35 wherein the silicon containing
semi-conductor electrode forms at least a portion of the floor of the
micro flow chamber and at least a portion of the wells for retaining
living cells are defined by the silicon containing semi-conductor
electrode.

37.   The apparatus of Claim 34 wherein the means for retaining
living cells in the micro flow chamber comprises a biologically
compatible adhering agent capable of adhering the living cells to a
surface of the micro flow chamber.

38.   The apparatus of Claim 37 wherein the biologically
compatible adhering agent is agarose.

39.   The apparatus of Claim 34 wherein the means for retaining
living cells in the micro flow chamber comprises a compartment in a
surface of the micro flow chamber and a membrane permeable to the
cell affecting agent, the permeable membrane being positioned such
that it covers the compartment and retains the living cells therein.

40.   The apparatus of Claim 34 wherein the means for detecting
the effect of the cell affecting agent comprises a silicon
semiconductor electrode in contact with or in the immediate vicinity
of the living cells.

41.   The apparatus of Claim 34 wherein the means for retaining
living cells in the micro flow chamber comprises a membrane or
microcarrier to which the living cells are adhered.

Fig.1

Fig.2

LASER LIGHT

Fig.3

INLET TUBE 3

SILICON RUBBER GASKET

SILICON RUBBER GASKET 11

KELEF COVER SLIP RETAINER 12

OUTLET TUBE 4

KELEF BODY

BRASS MOUNTING FLANGE

GLASS COVER SLIP 8

SILICON 2

BRASS BASE PLATE

HEATER PLATE 10

Fig.4

CONTROLLING
ELECTRODE 13

MEDIUM
IN 3

MEDIUM
OUT 4

2

COVER
SLIP 8

MICRO-
MACHINED
WELLS 7

1 cm
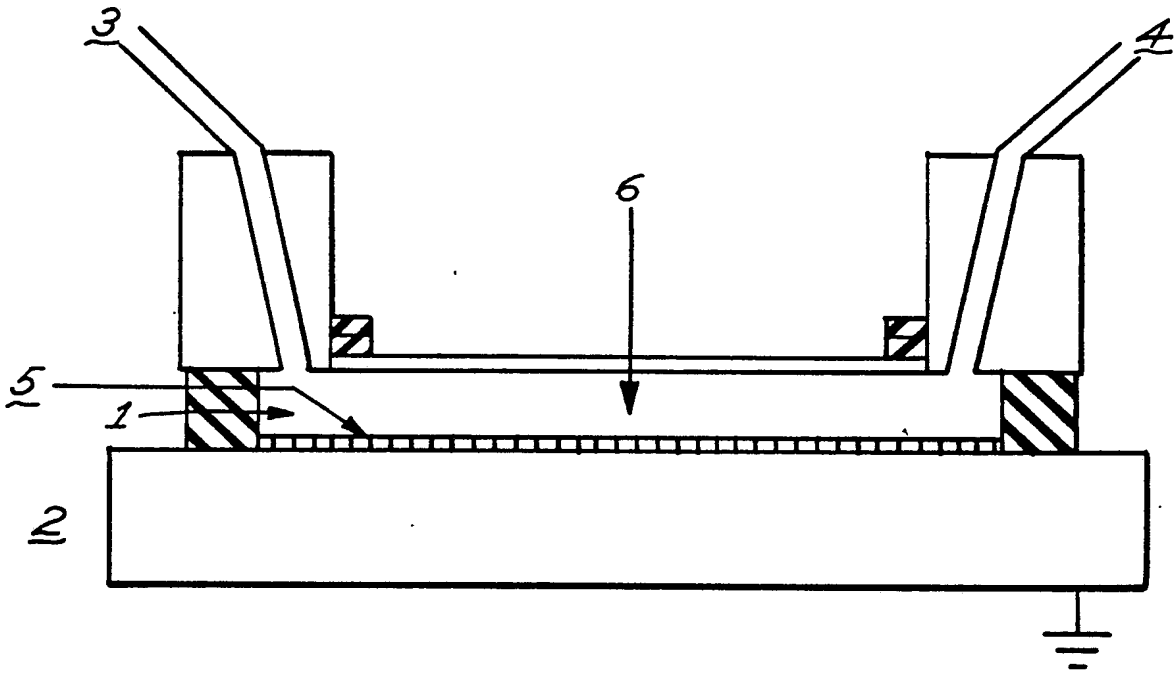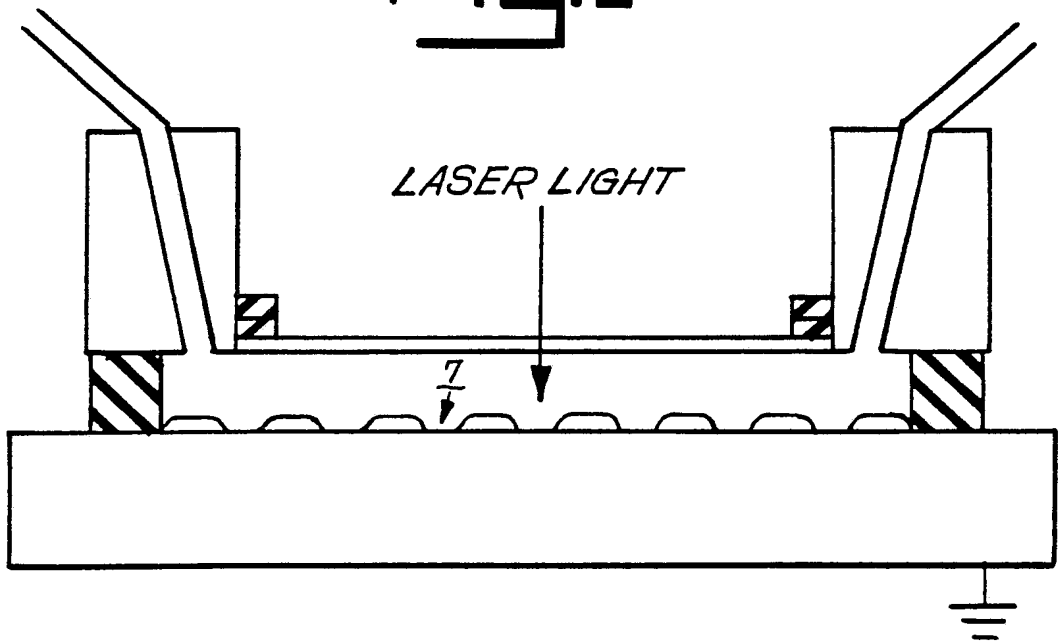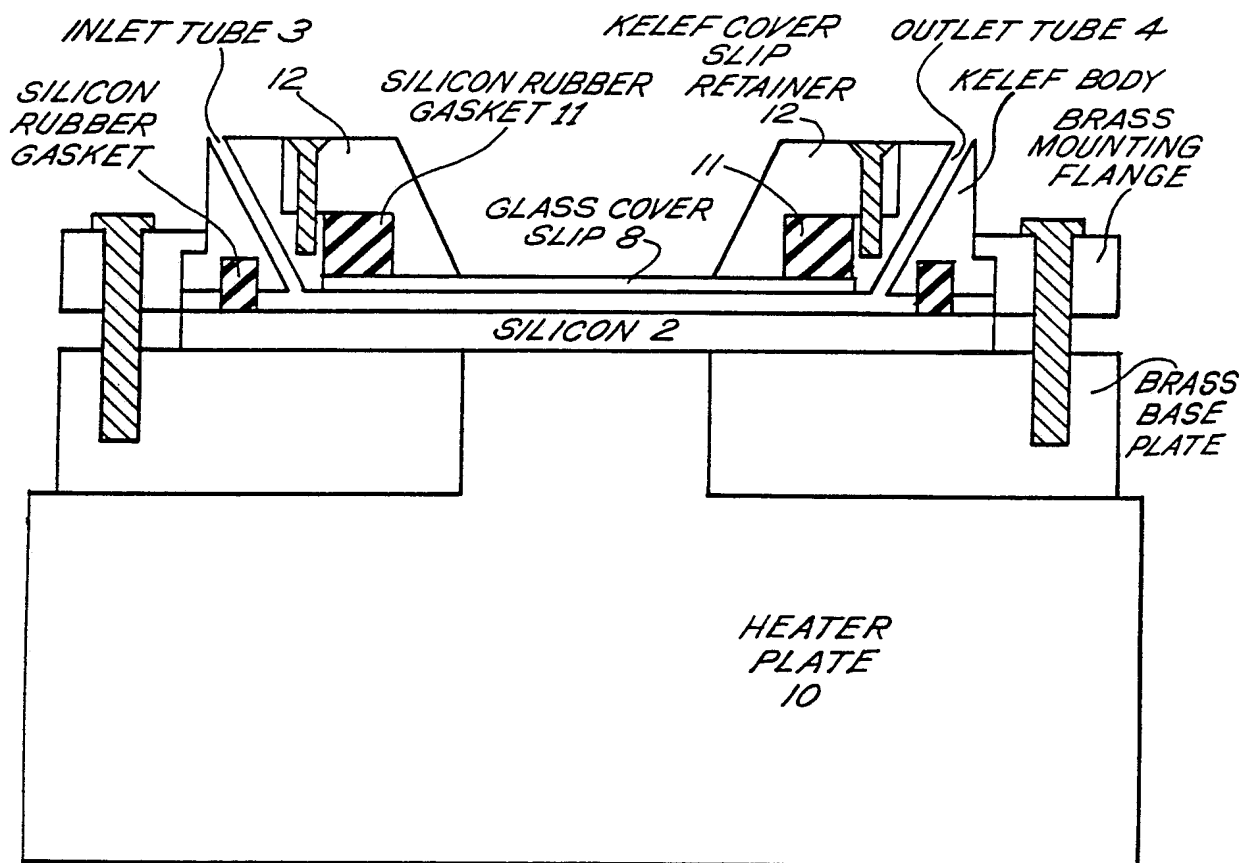
TEFLON SPACER
WITH CHANNEL
CUTOUT 14
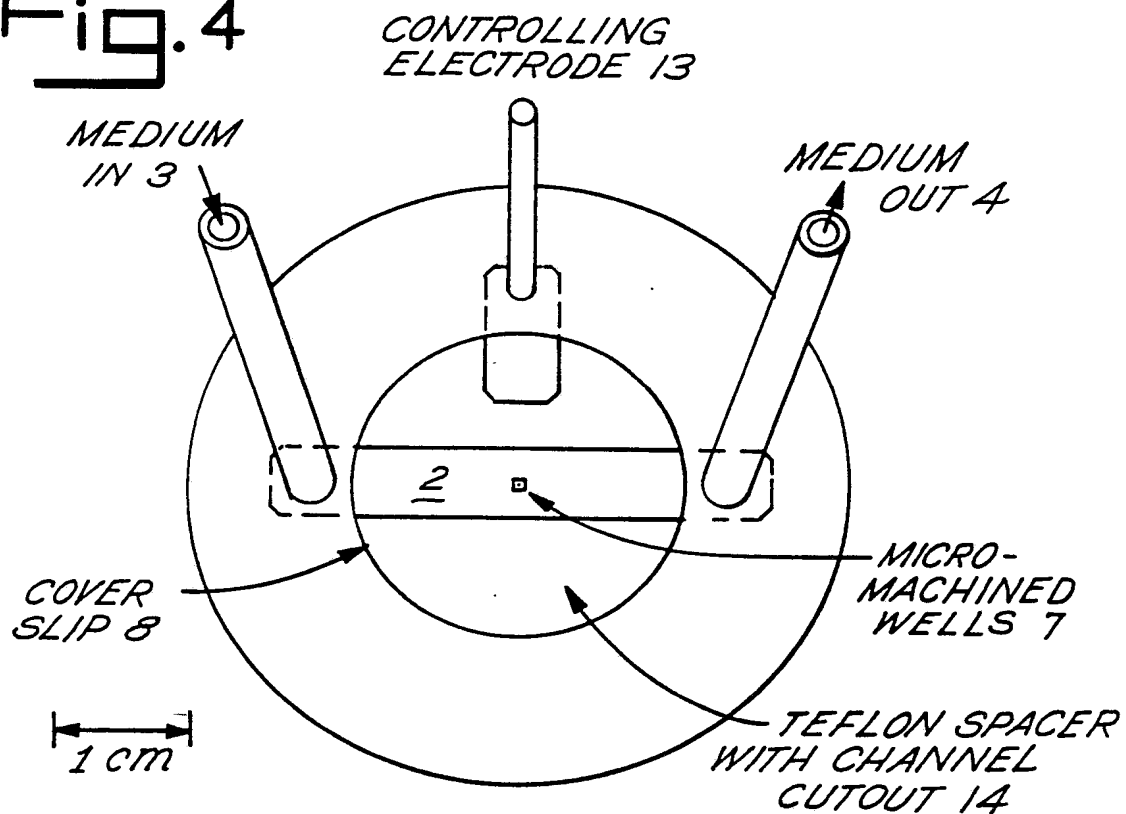
Fig.5

CELL AFFECTING AGENT SYSTEM CONFIGURATION

SYRINGE PUMPS → DEGASSERS → INJECTION LOOPS → FLOW CHAMBER → REFERENCE ELECTRODE RESERVOIR

SOFTWARE → COMPUTER ↔ ELECTRONICS

FLOW CHAMBER ↔ ELECTRONICS

REFERENCE ELECTRODE RESERVOIR → DRAIN

Fig.6
DEGASSER

VACUUM GUAGE

VACUUM CHAMBER

OUTLET HOSE

GAS PERMEABLE TUBE

ON/OFF VALVE

HOLLOW CANNULA

SYRINGE

Fig.7

Cover slip

Cells

→Culture medium →

100μm

Adherent Cells on Cover Slip
or on Sensor Surface

Fig. 8

Cover slip

→ Culture medium →

100μm

Fig. 9

SSI
R.E.   C.E.

SI SENSOR

R4
1000

R3
49.9K

+12

3   8   U2A
2   —
     4   OPA-2111 KP
1

-12

R2
750

6   U2B
5   7   50□V/μA
         +
         OPA-2111 KP

R1
750

U1

15 14   VREF+
         VREF-
                 +12

                 V+   20

11 12   B0-L
10   B1   10   18
9   B2
8   B3   10   19
7   B4
6   B5
5   B6
4   B7
3   B8   COMP   16
2   B9
         B10
1   B11-M   V-   17
13   VLC

C1
1NF

-12

C2
100 NF

AM 6012 PC
0-2nA PER OUTPUT <> +/- 1.5 VOLT SWEEP

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Fig.10
Determination of Metabolic Rates

BARS INDICATE PERIODS IN WHICH FLOW OF MEDIUM IS OFF

Fig.11

EFFECTS OF IRRITANTS ON METABOLIC RATE OF KERATINOCYTES
(MEASUREMENT DURING PRESENCE OF IRRITANT)

Fig.12

EFFECTS OF IRRITANTS ON METABOLIC RATE OF KERATINOCYTES
(DEMONSTRATION OF RECOVERY)
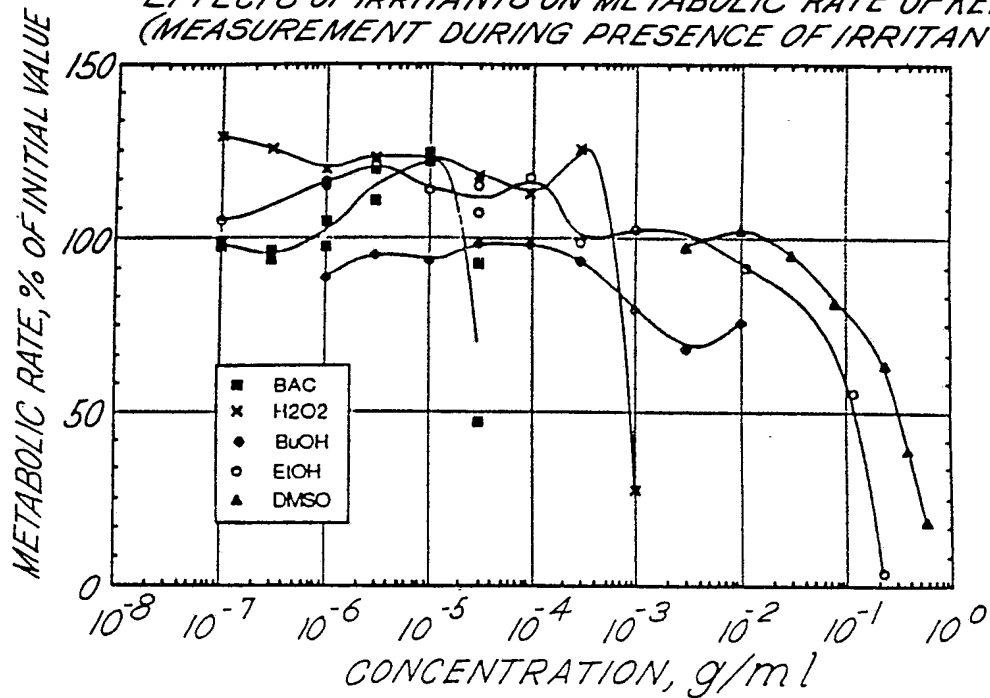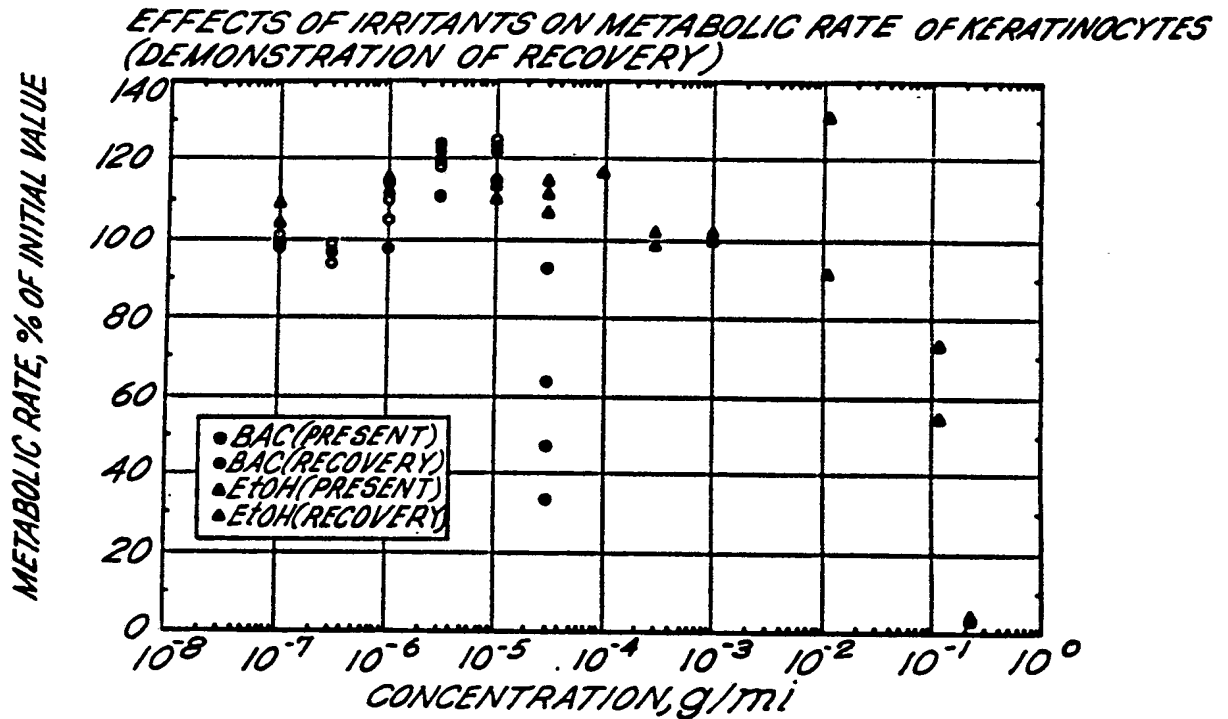
Fig.13

Recovery from Exposure to Ethanol and DMSO

Fig.14

Effect of Valinomycin on L Cells

t, min

add valinomycin

Fig.15

Effect of 100 μM Valinomycin on Fibroblastic (L) Cells

Start Valino-mycin

Stop Valino-mycin

Time (min)

Fig. 16

Response of MDCK Cells on Si to PMA

Legend:
- Steady-State Psi, mV
- Acidification Rate, -pH/min
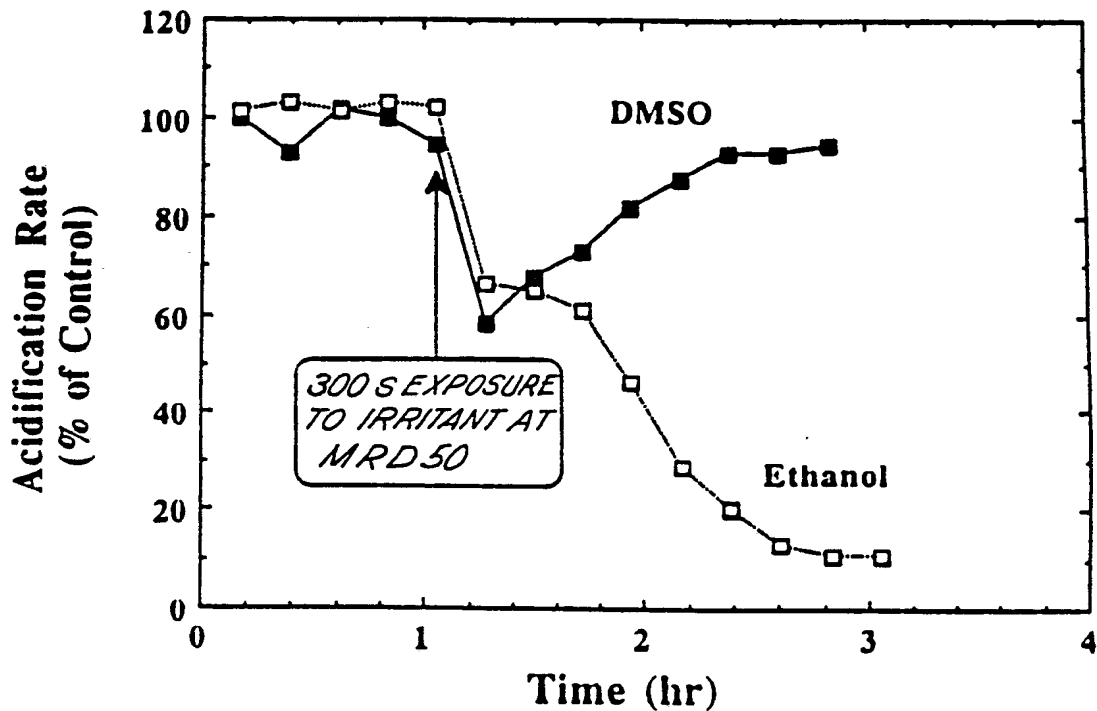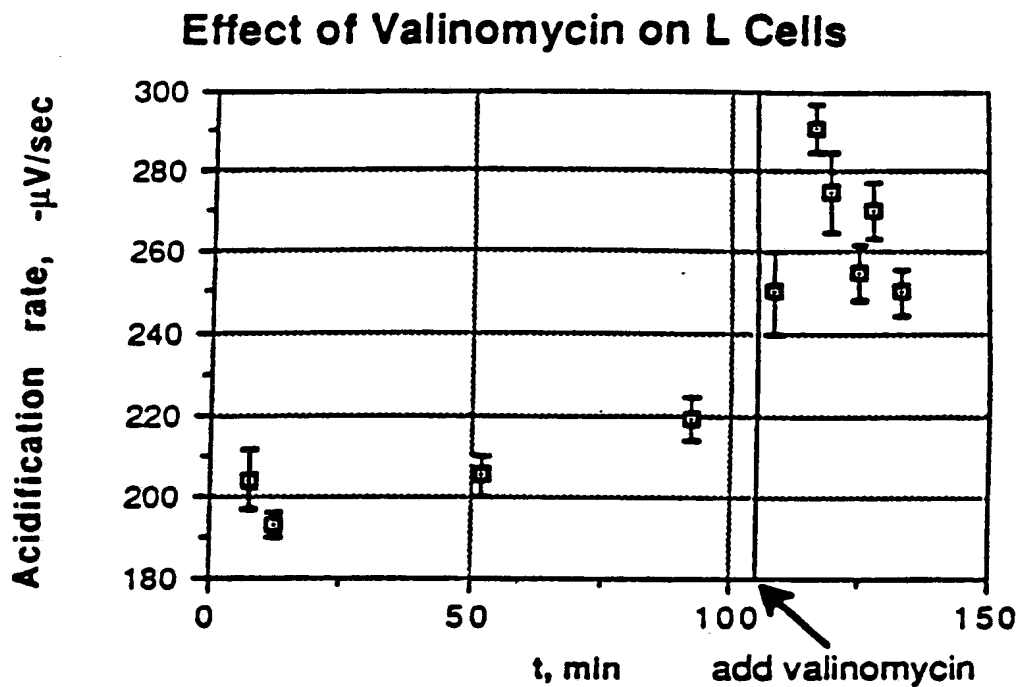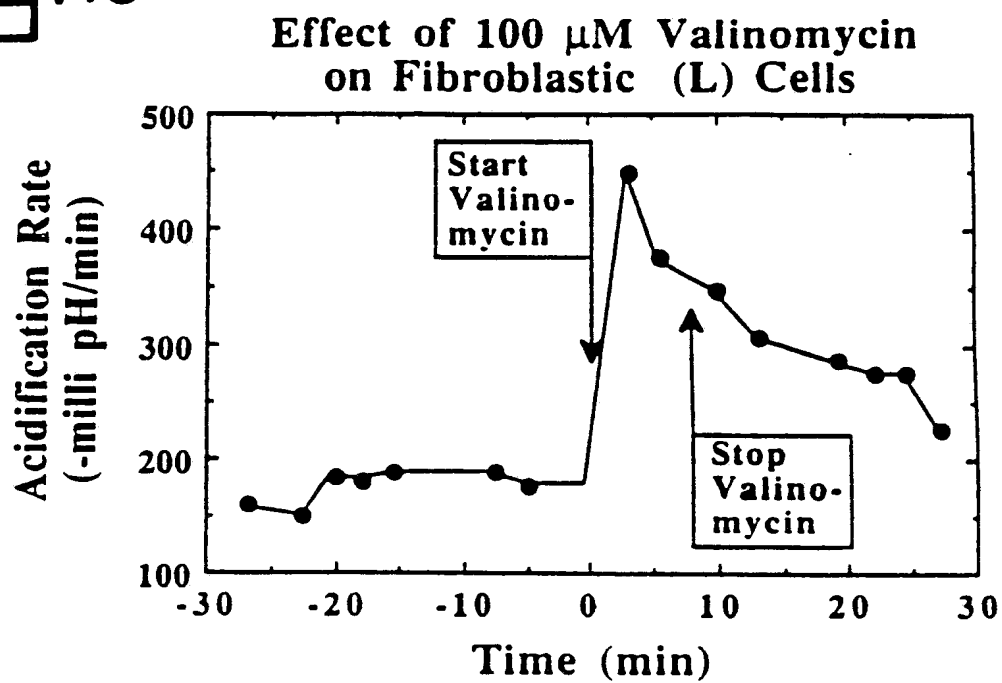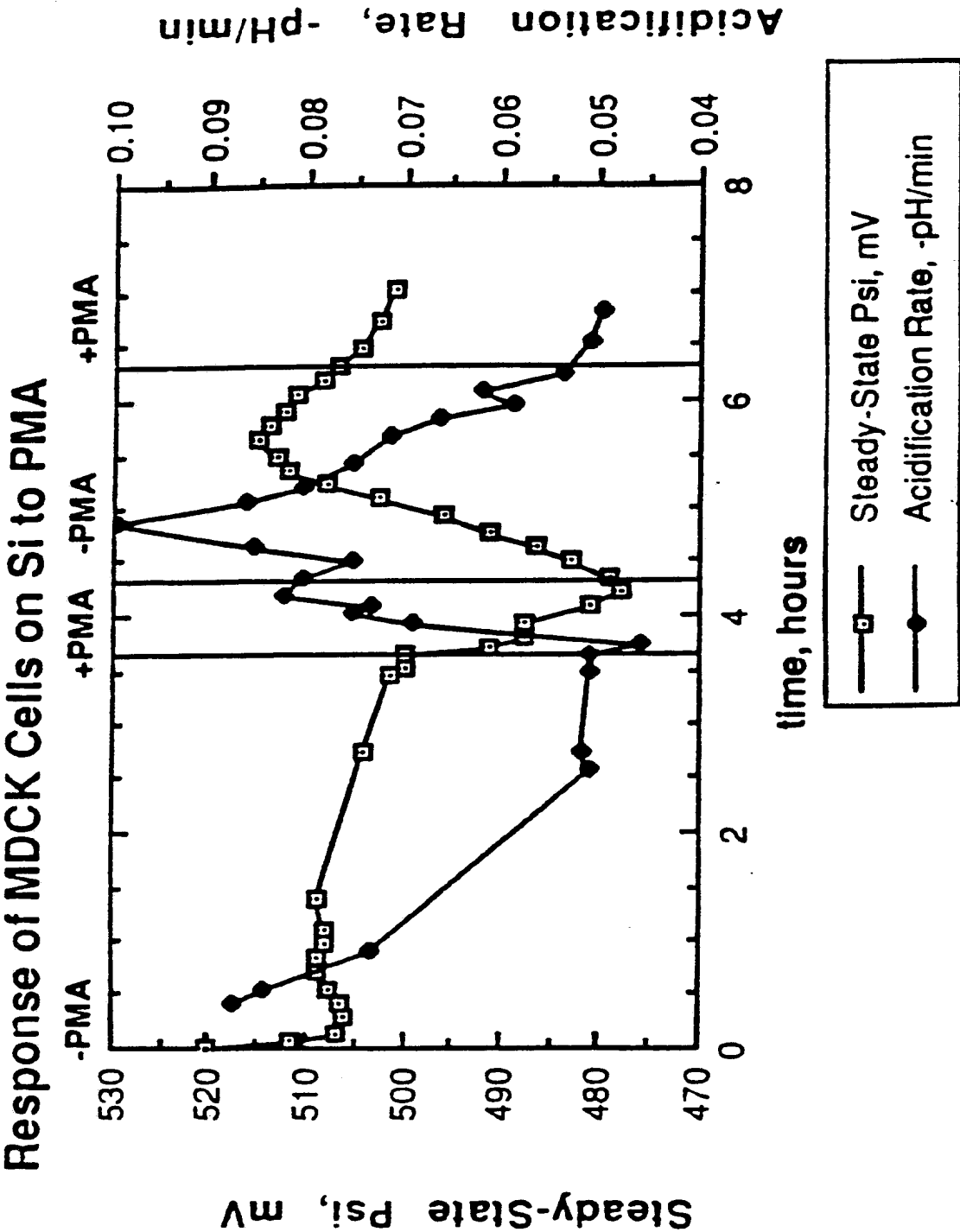
Fig.17

Effect of LPS on BHK Cell Metabolic Rate

Fig. 18

MDCK on Si: epi effect

med only
epi(3uM)

Acidification Rate, pH/min

time (hrs)
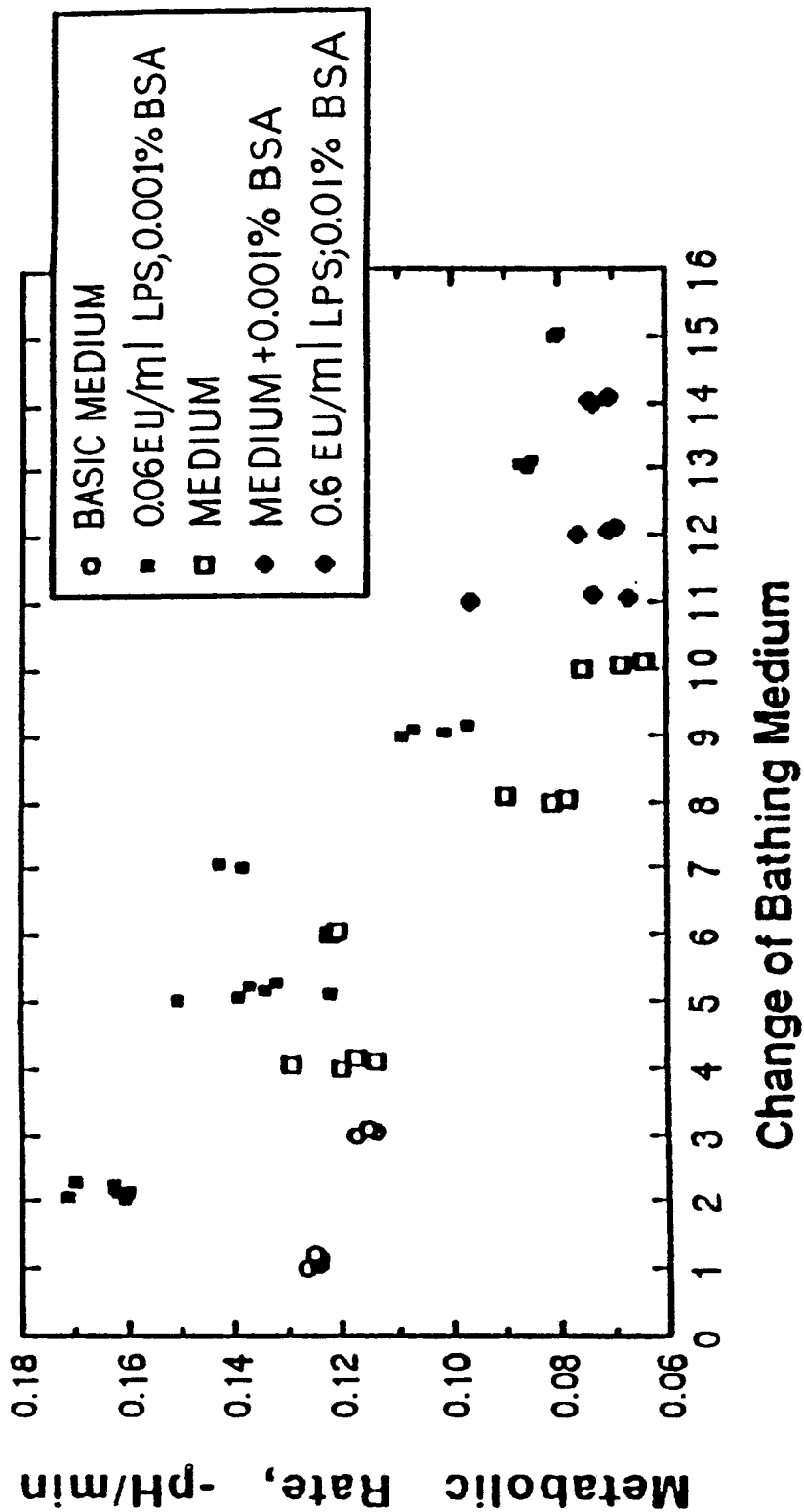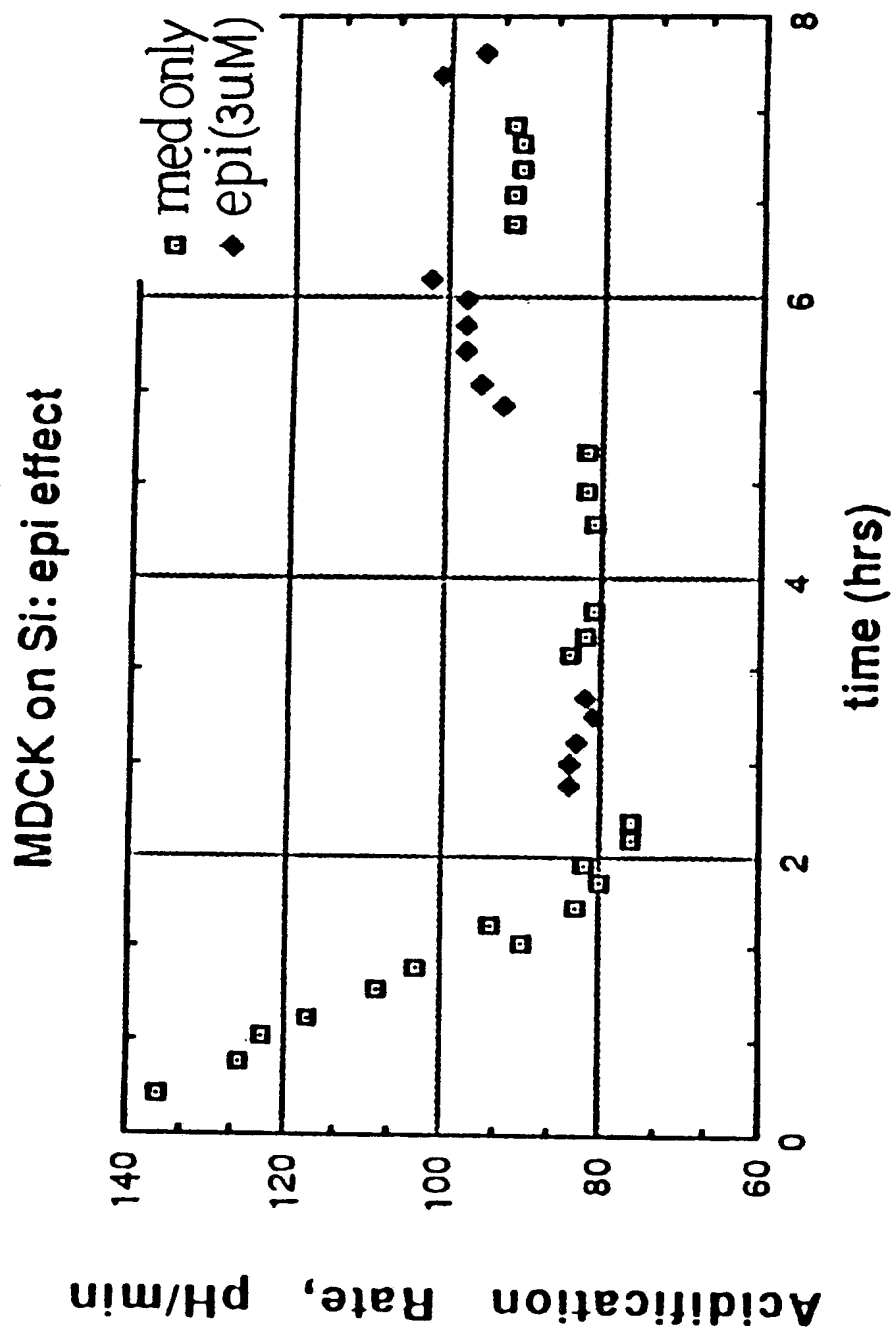
Fig.19

Effect of Epinephrine on MDCK Cells
Immobilized in Agarose
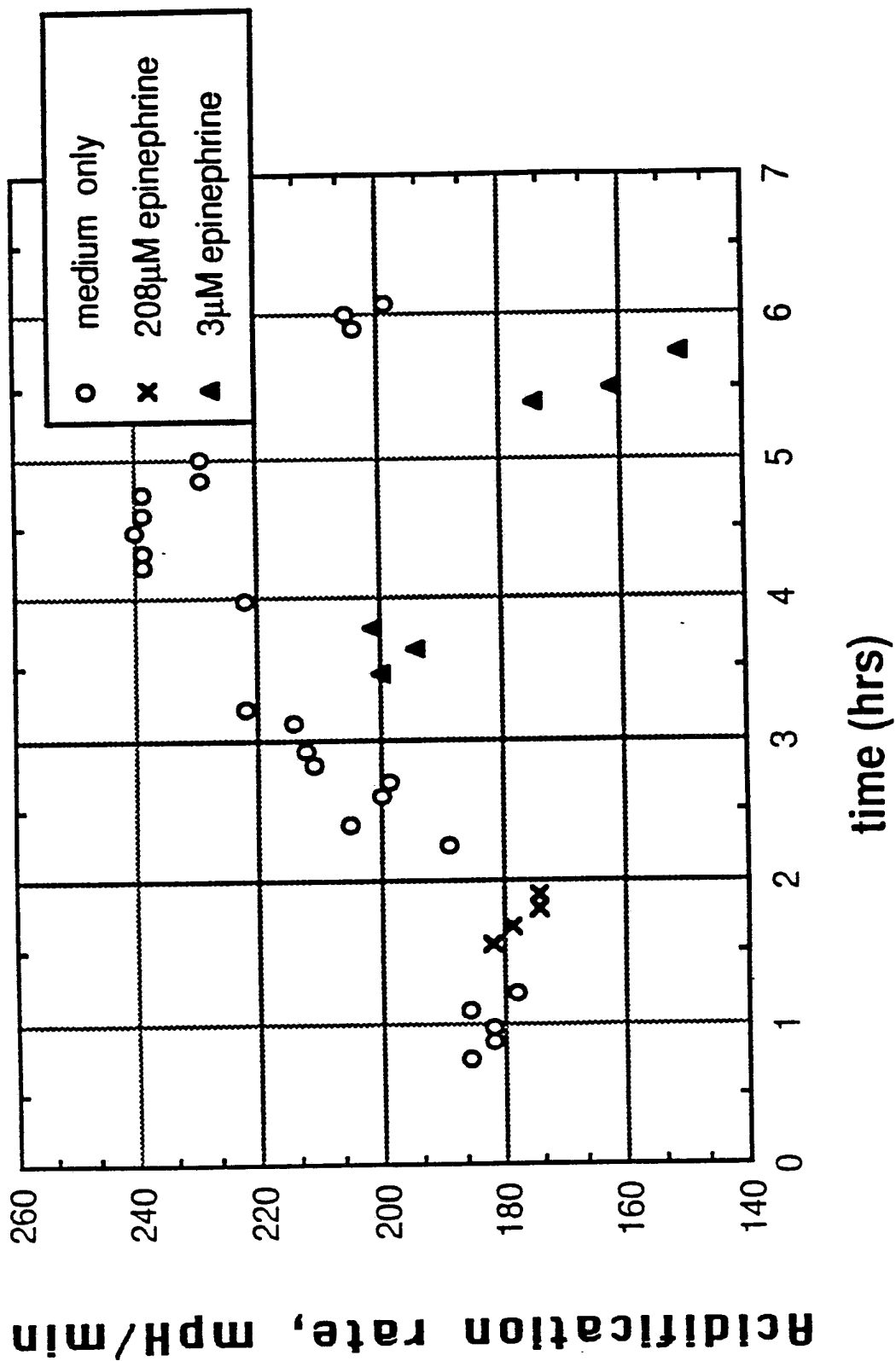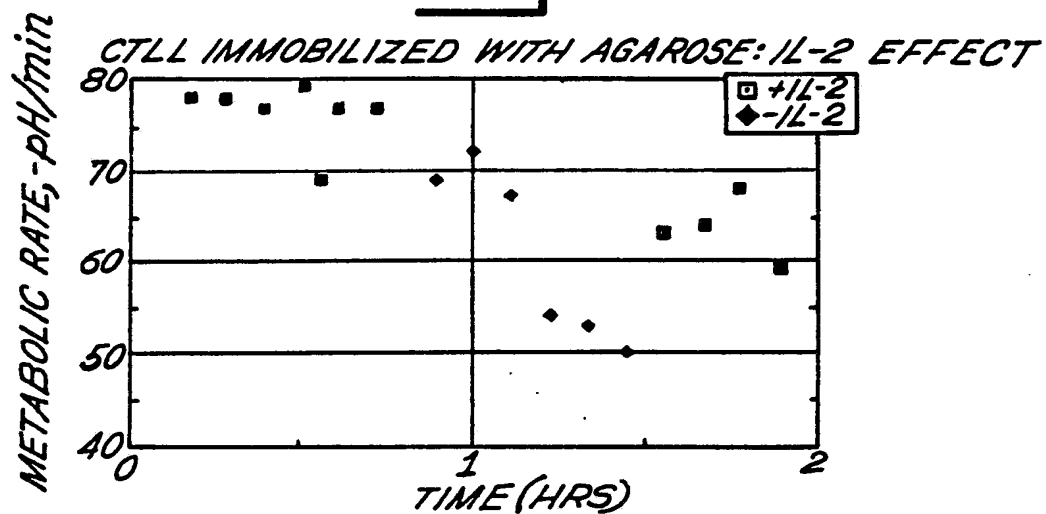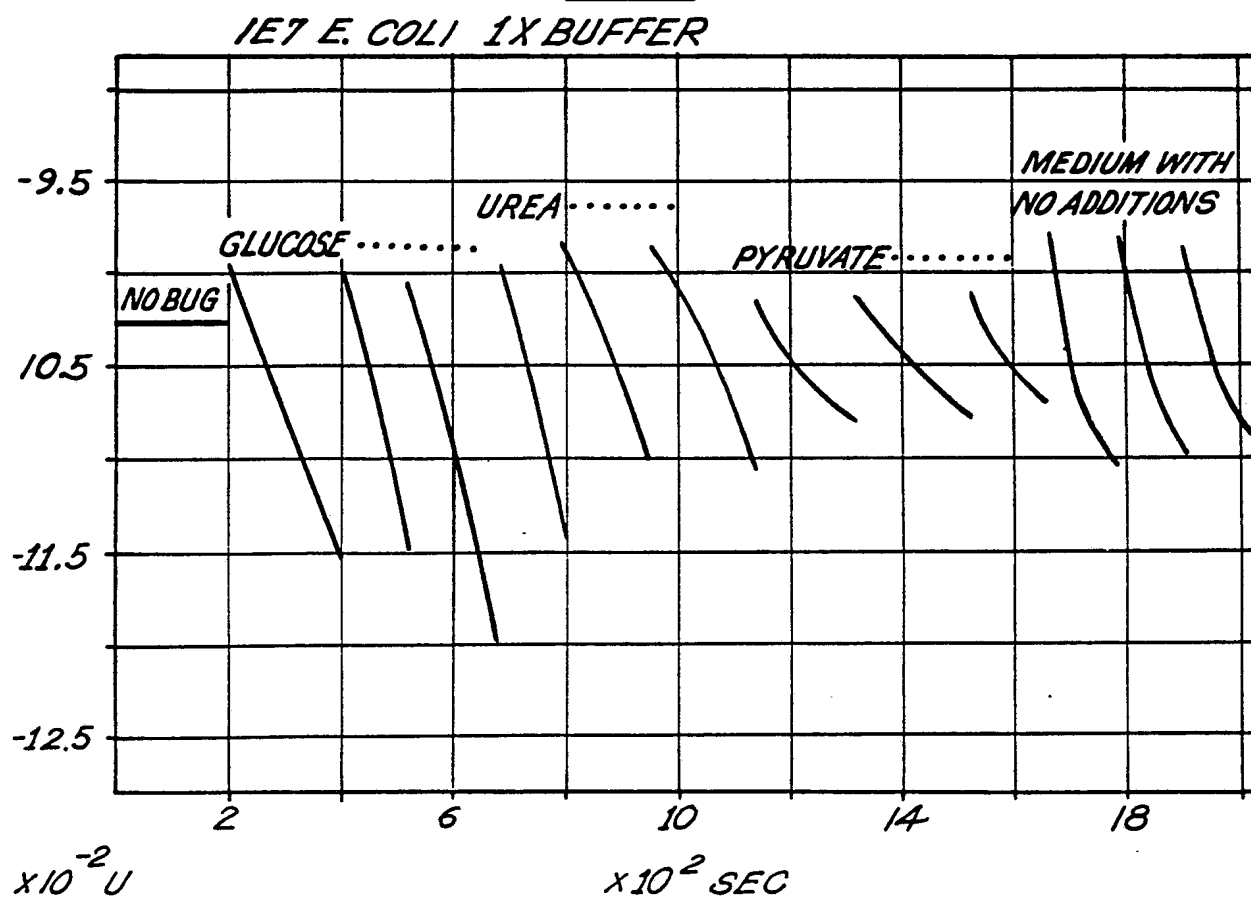
Legend:
- ○ medium only
- ✕ 208μM epinephrine
- ▲ 3μM epinephrine

Acidification rate, mpH/min

time (hrs)

Fig.20

CTLL IMMOBILIZED WITH AGAROSE: IL-2 EFFECT

Legend:
- □ +IL-2
- ◆ -IL-2

Y-axis: METABOLIC RATE, -pH/min (40 to 80)
X-axis: TIME (HRS) (0 to 2)

Fig.21

IE7 E. COLI 1X BUFFER

NO BUG

GLUCOSE · · · · · · ·

UREA · · · · · · ·

PYRUVATE · · · · · · ·

MEDIUM WITH NO ADDITIONS

Y-axis: -9.5, 10.5, -11.5, -12.5

X-axis: 2, 6, 10, 14, 18

$x10^{-2} U$ $x10^{2} SEC$

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Fig.22

1E7 pH 1X BUFFER

Fig.23

RATE OF pH CHANGE VS NUMBER OF E. COLI

Fig.24

0.45
0.44
0.43
0.42
0.41
0.40
0.39
0.38
0.37
0.36
0.35

A   *B*

VOLTS   1303.0 SEC/DIV

Fig.25

SLOT WELLS IN A STAINLESS STEEL FOIL

3   *3*

1

2

FLOW ---->   ⊢———⊣
100 µm
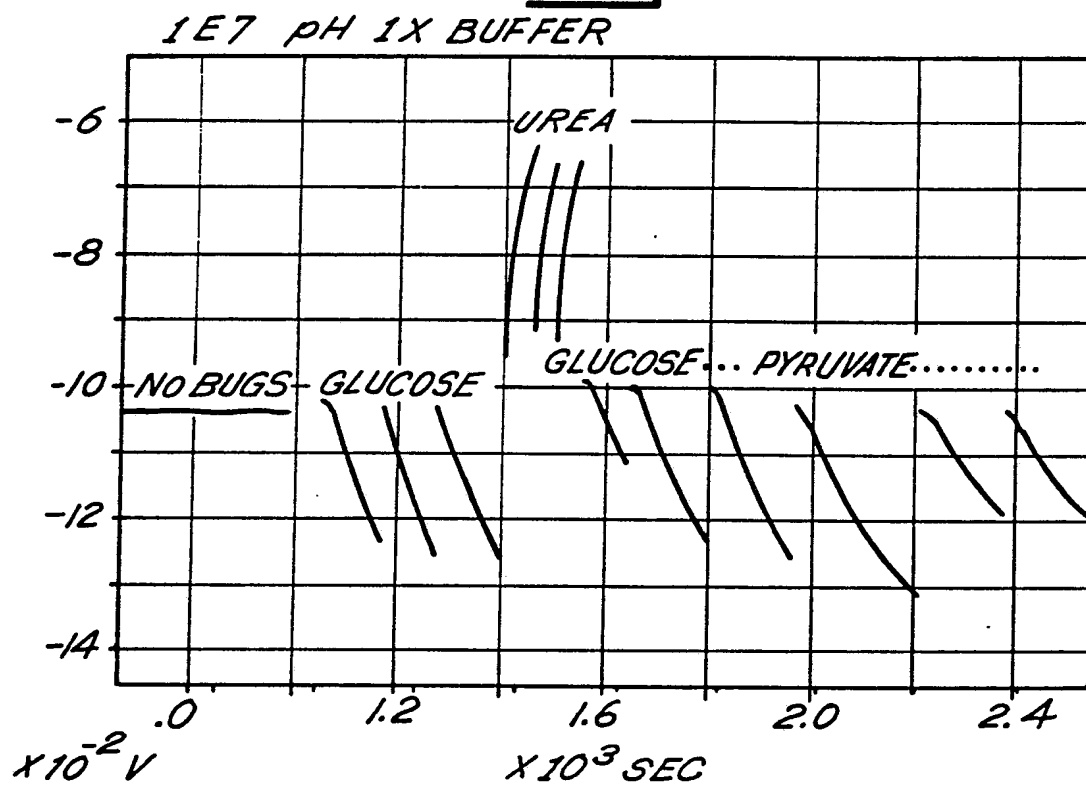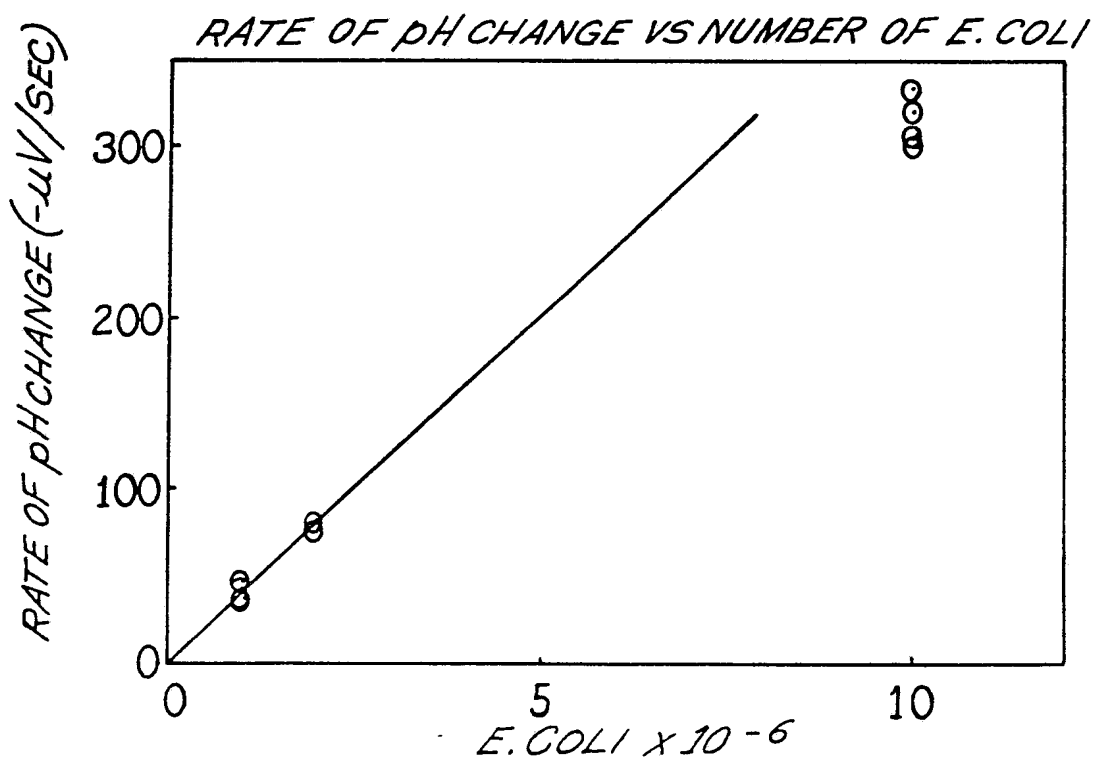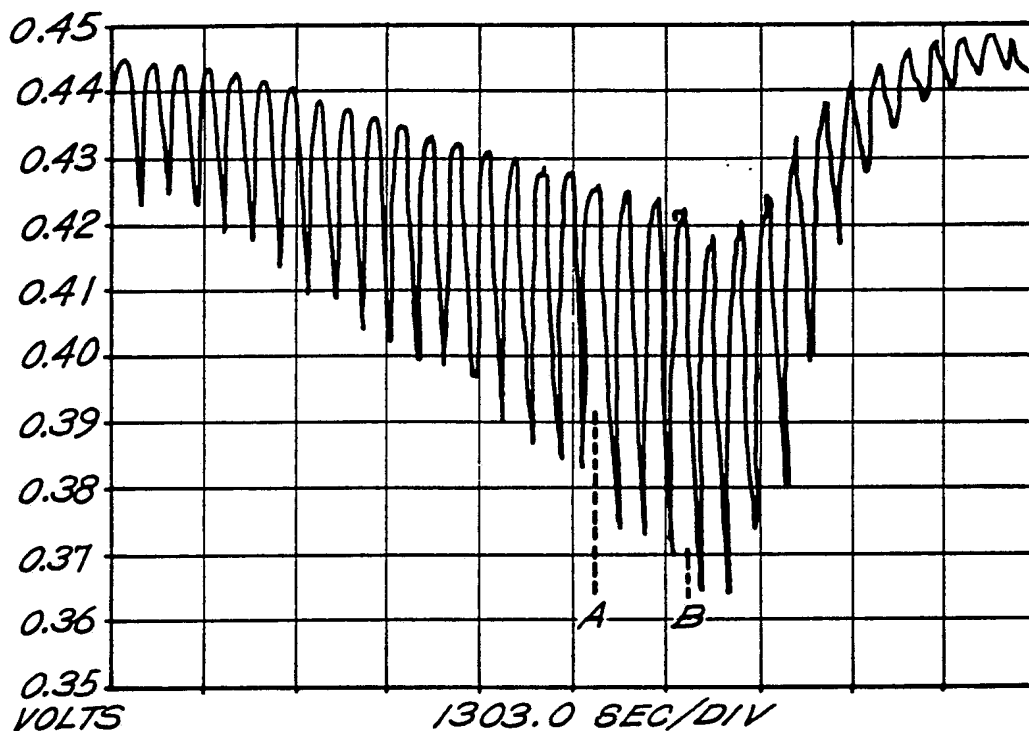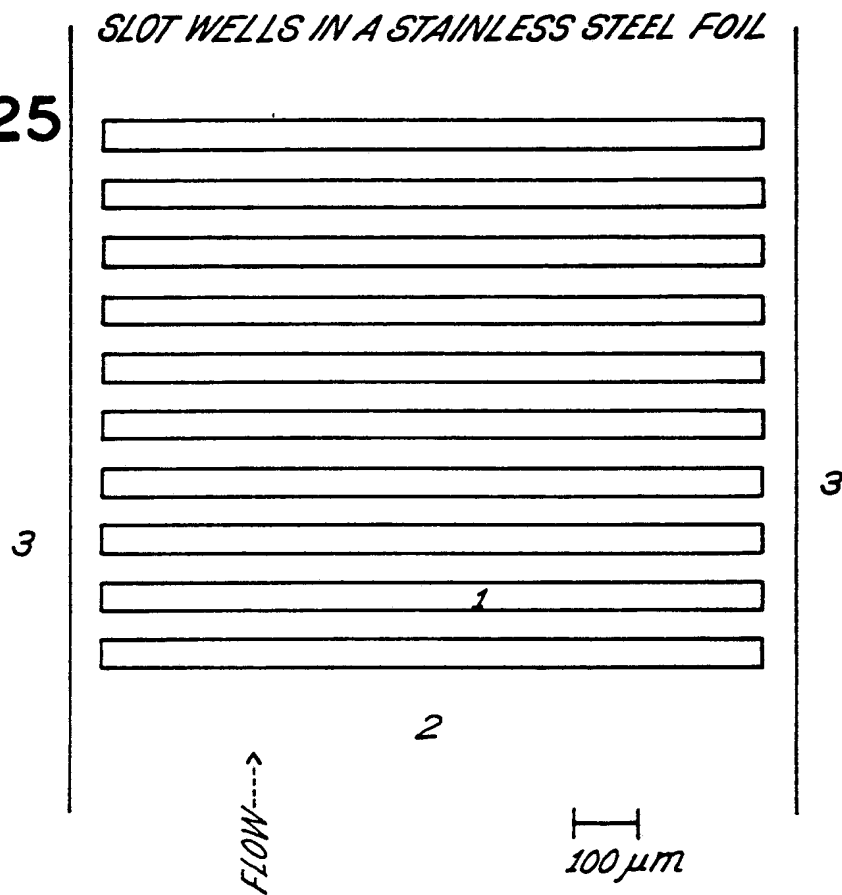
Fig.26

Isoprolerenol Dose Response on FBS
Starved 821 Cells
7-14-89

control
0.01 µM Iso
0.1 µM Iso
0.5 µM Iso
1 µM Iso
5 µM Iso
10 µM Iso
100 µM Iso

% Metabolic Rate at Time Zero

Time (hrs)
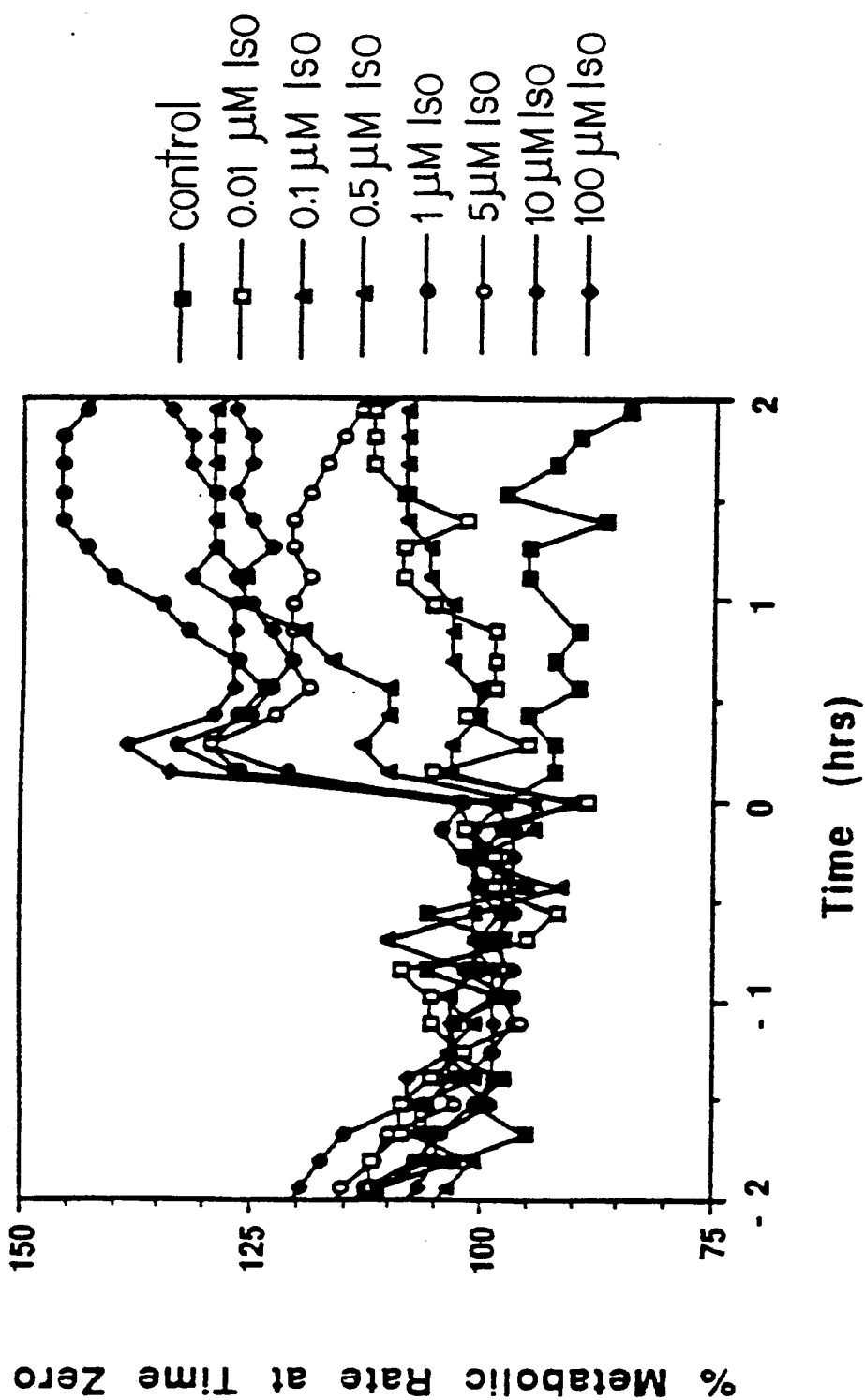
Fig.27

EFFECT OF VARIOUS HORMONES ON MIC2 CELLS
6/23/89

Fig.28

Effect of 10 mM Carbachol, 10 μM Atropine,
10 mM Carbachol + 10 μM Atropine on MIC1
Cells Starved from FBS

6-20-89

control
carbachol
atropine
carbachol+atropine

% of Metabolic Rate at Time Zero

time (hrs)
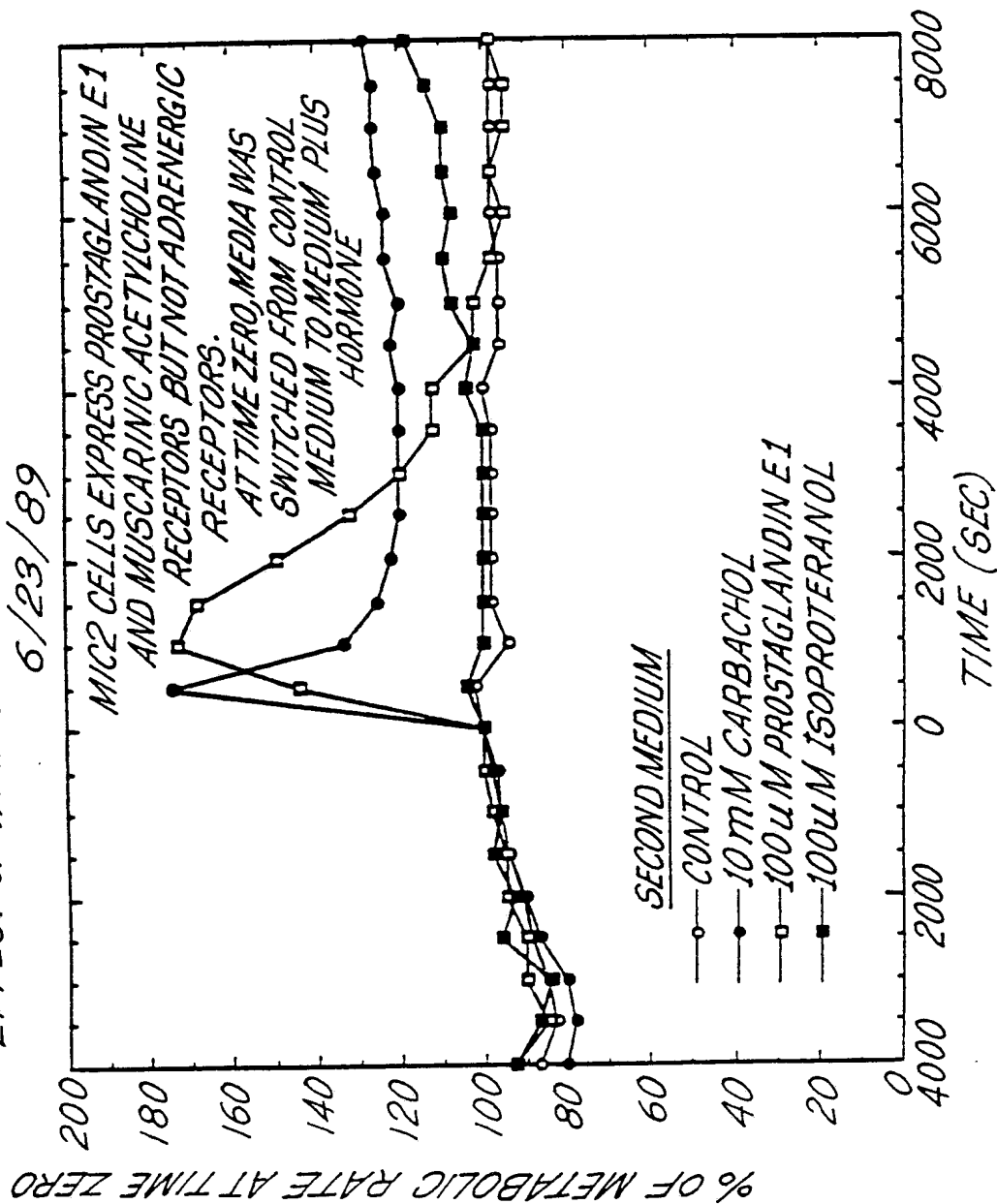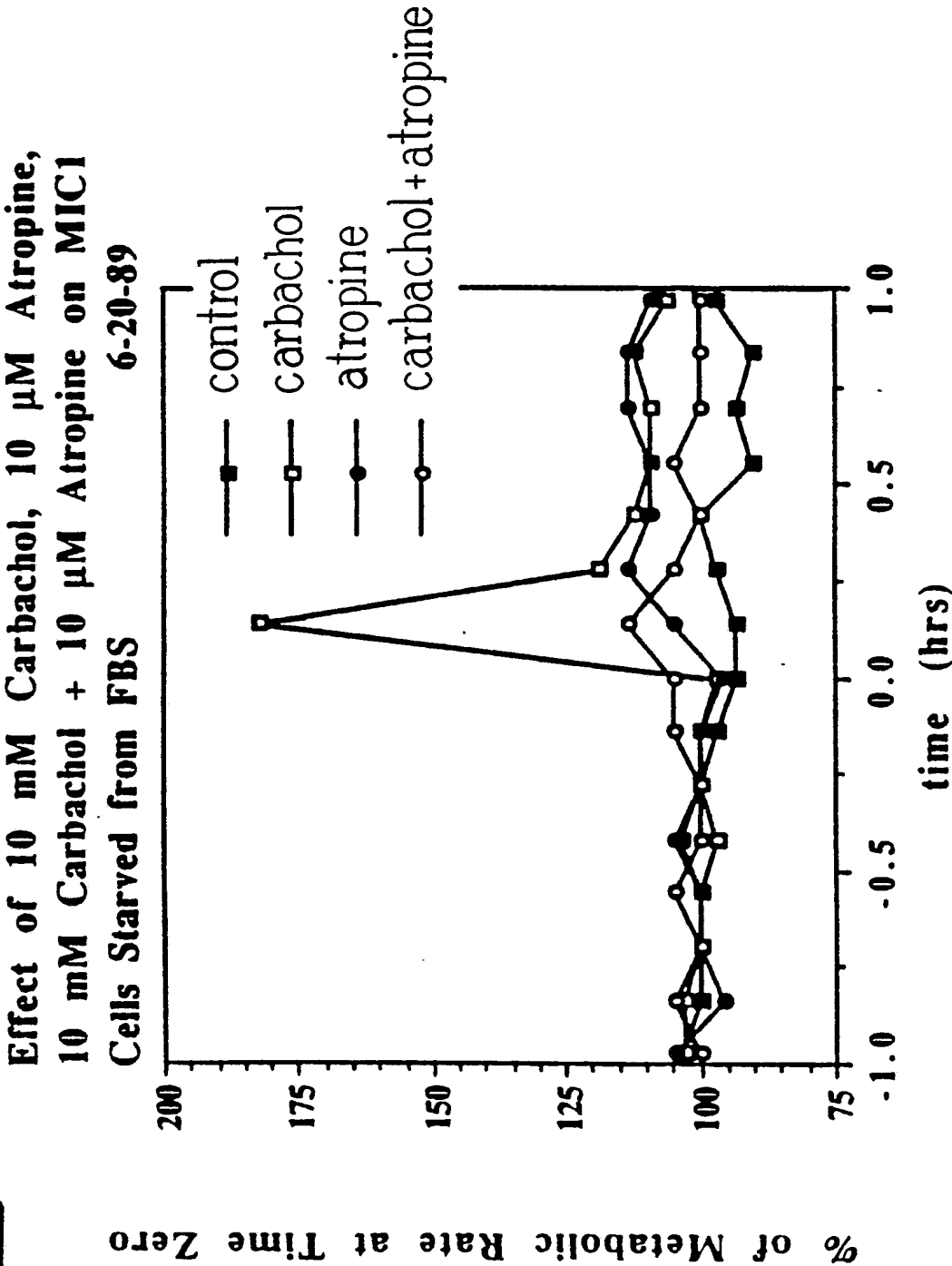
Fig.29

Time Course of Carbachol Stimulation of MIC1 Cells
Vpip vs.time, 6/20/89

PUMP WAS TURNED OFF AT TIME ZERO.
AT TIME ZERO, CELLS WERE EXPOSED TO
10 mM CARBACHOL FOR LESS THAN 30 SECONDS.

TIME (SEC)

V pip-Vpip (W/FLOW ON) (V)

- ○ CONTROL (INLET)
- ● CONTROL (OUTLET)
- □ 10 mM CARBACHOL (INLET)
- ■ 10 mM CARBACHOL (OUTLET)

Fig.30

TIME COURSE OF CARBACHOL STIMULATION OF M1C1 CELLS METABOLIC RATE VS TIME, 6/20/89

PUMP WAS TURNED OFF AT TIME ZERO. AT TIME ZERO, CELLS WERE EXPOSED TO mM CARBACHOL FOR LESS THAN 30 SECONDS

CONTROL (INLET)
CONTROL (OUTLET)
10 mM CARBACHOL (INLET)
10 mM CARBACHOL (OUTLET)

TIME (SEC)

Fig.31

VSV INFECTION OF L CELLS—

VSV INFECTED

CONTROL

TIME (HR)

SUBSTITUTE SHEET

VSV Infection of Mouse L Cells
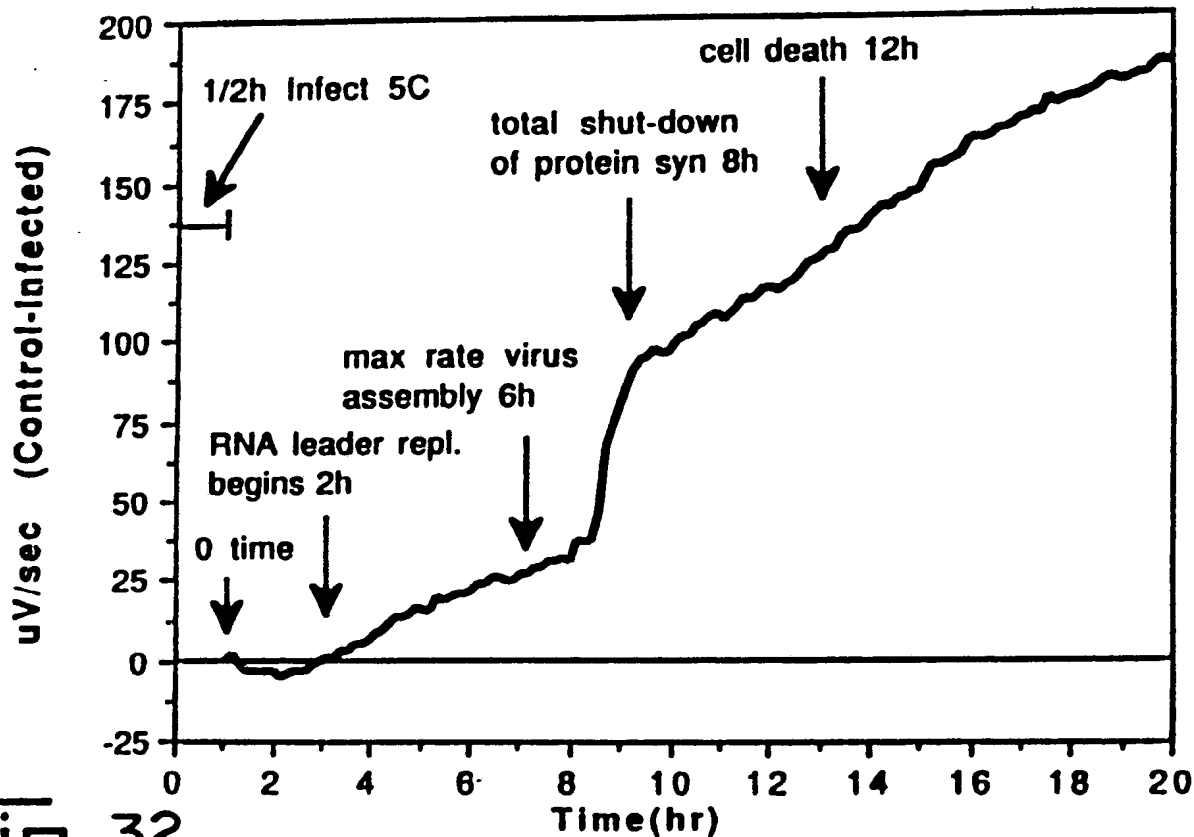
Fig.32

VSV Infection of L Cells-
Titration of Inoculum
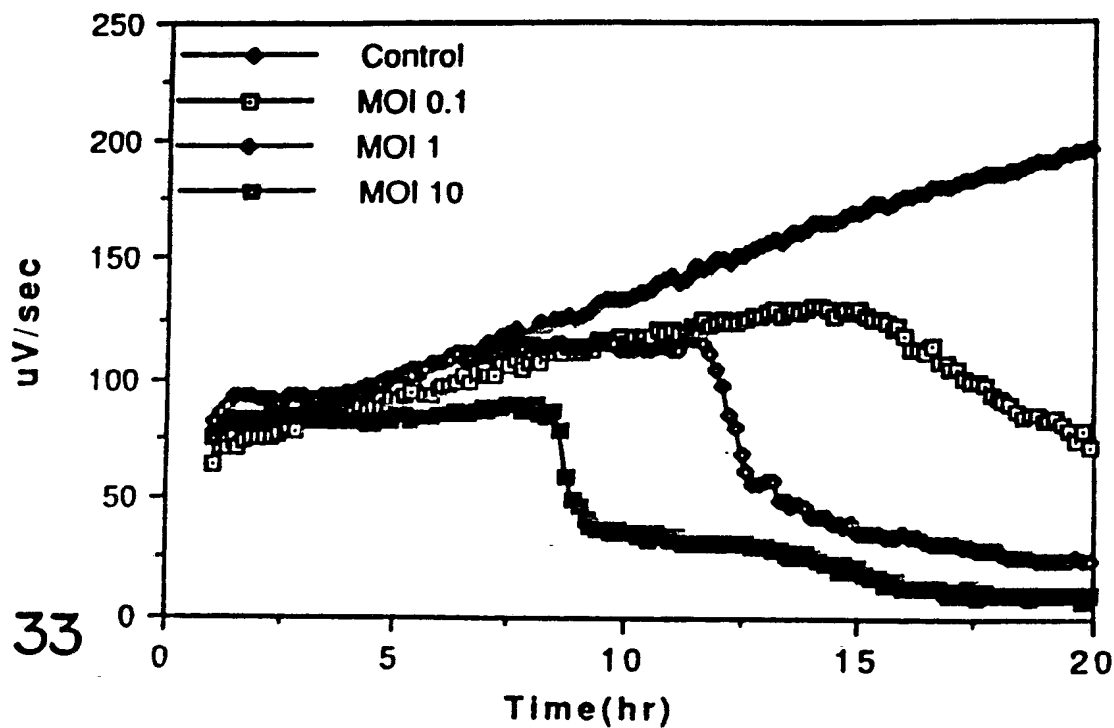
Fig.33

SUBSTITUTE SHEET

Fig.34

VSV Infection of L Cells-
Titration of Inoculum
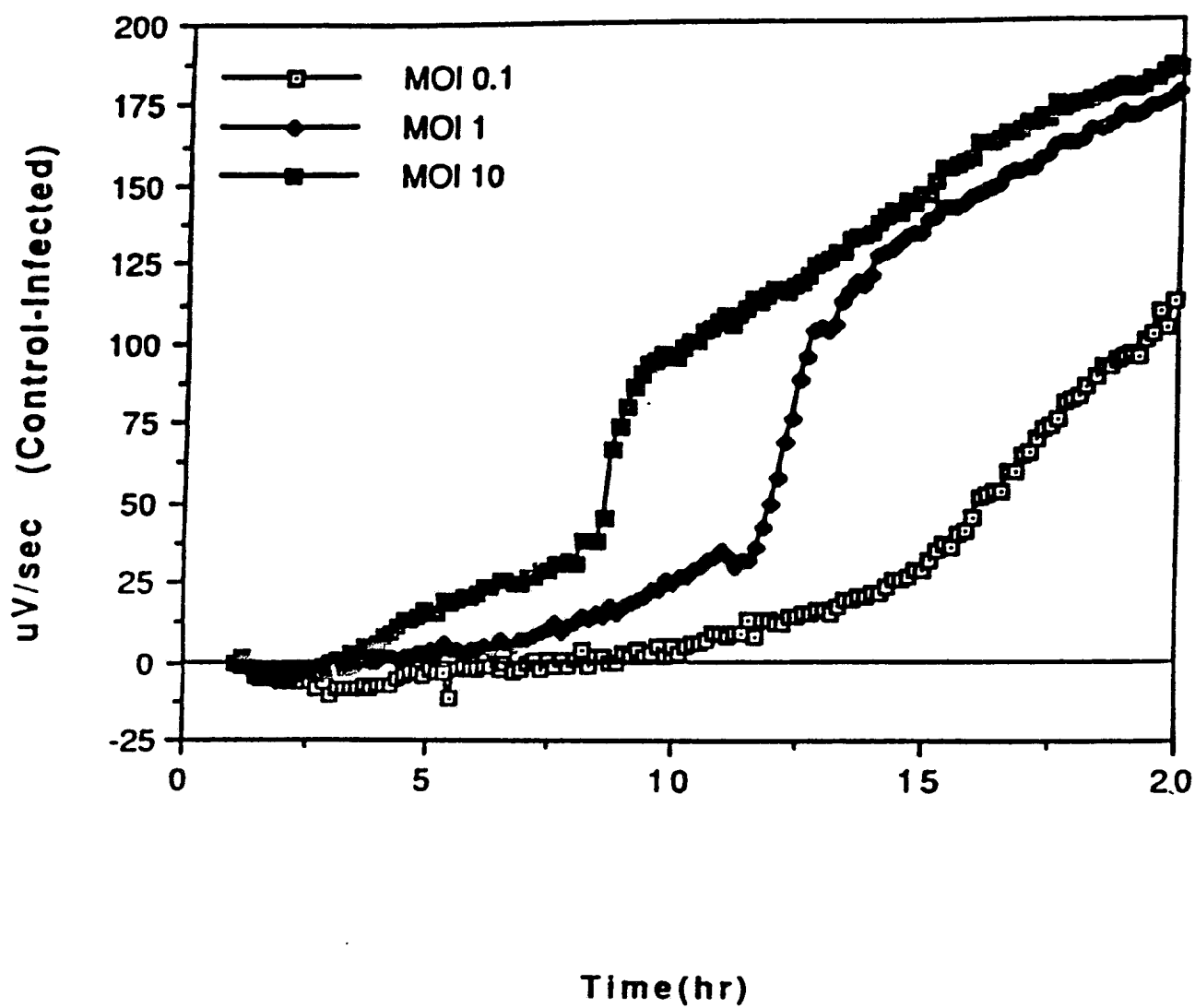
Fig.35

VSV Infection of L Cells-
UV Inactivation

Fig.36

VSV Infection of L Cells-
Antibody Neutralization

Fig.37A

Fig.37B

Fig.38

INTERFERON INHIBITION OF VSV INFECTION OF L CELLS
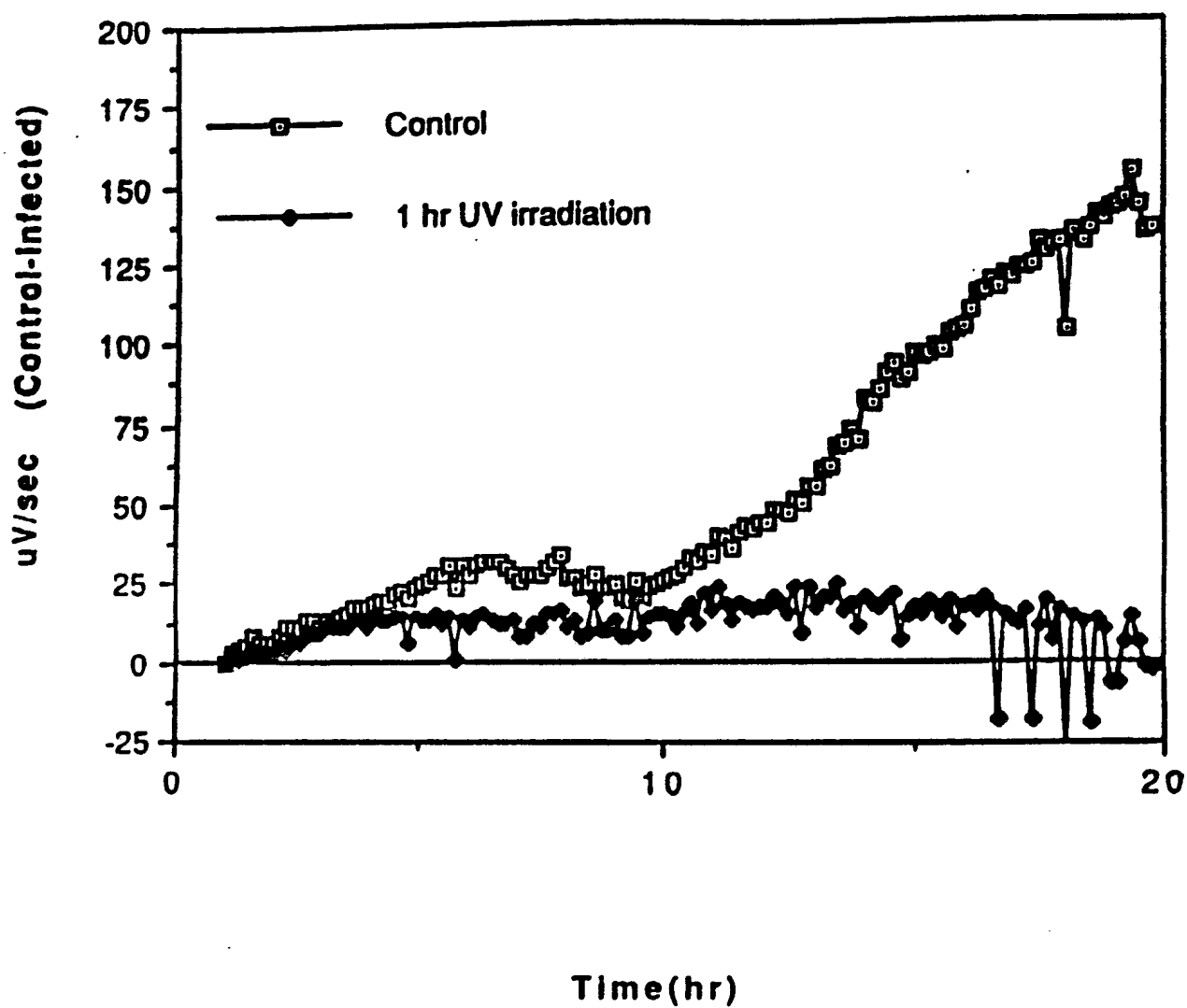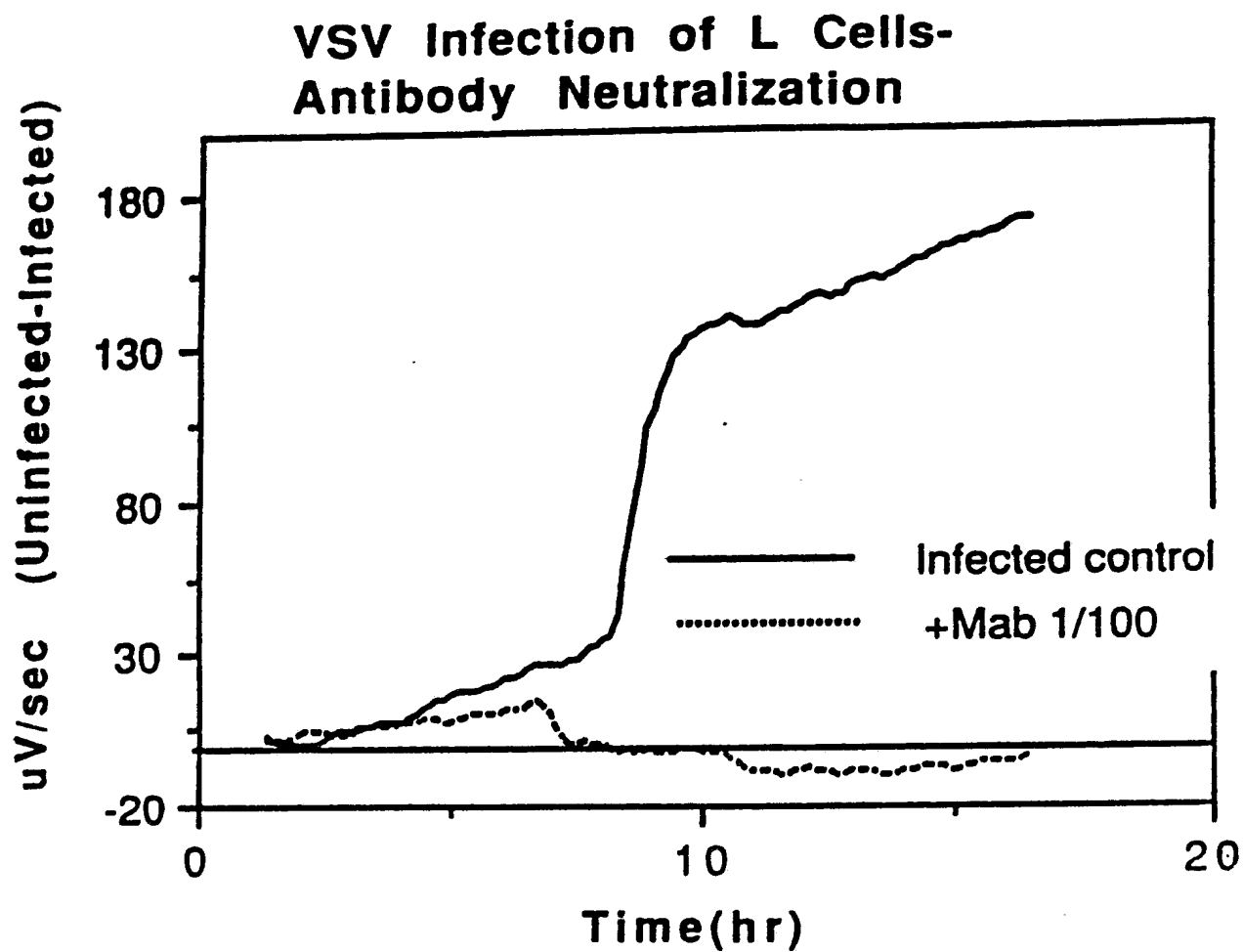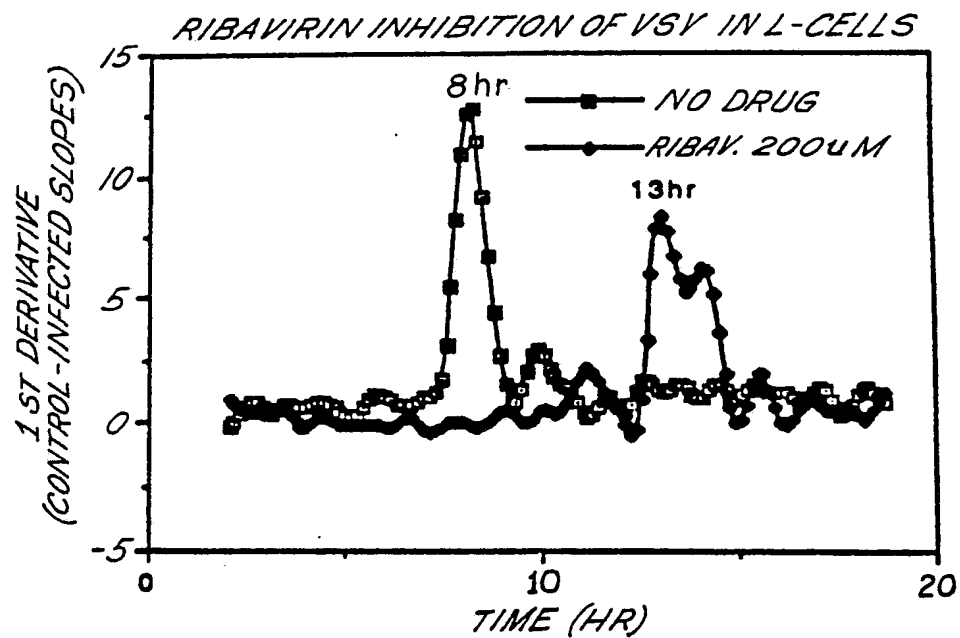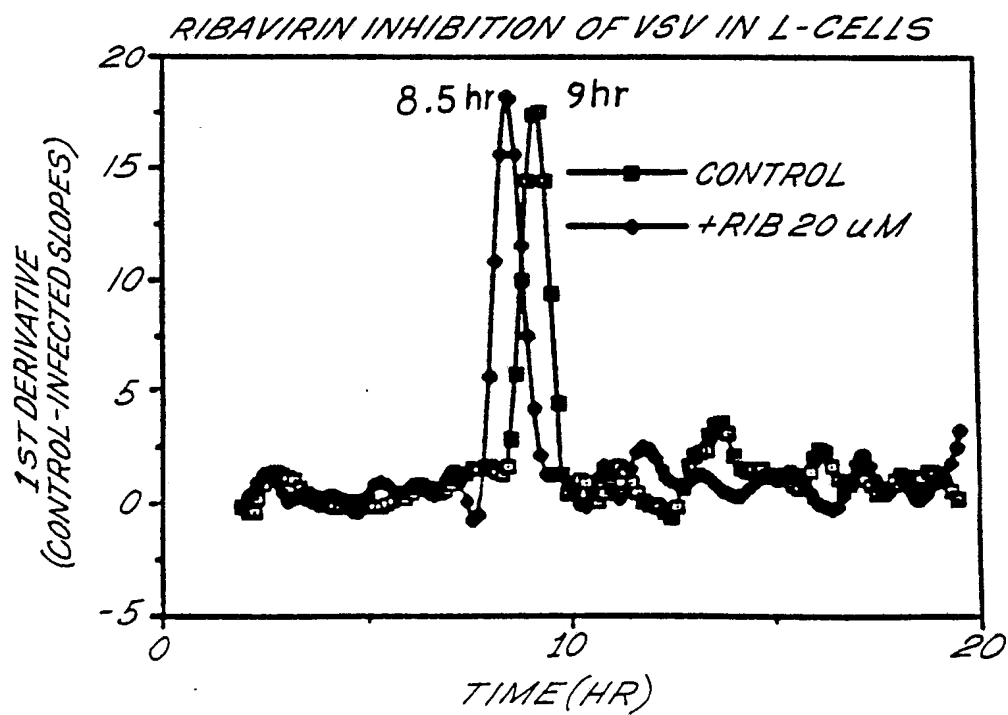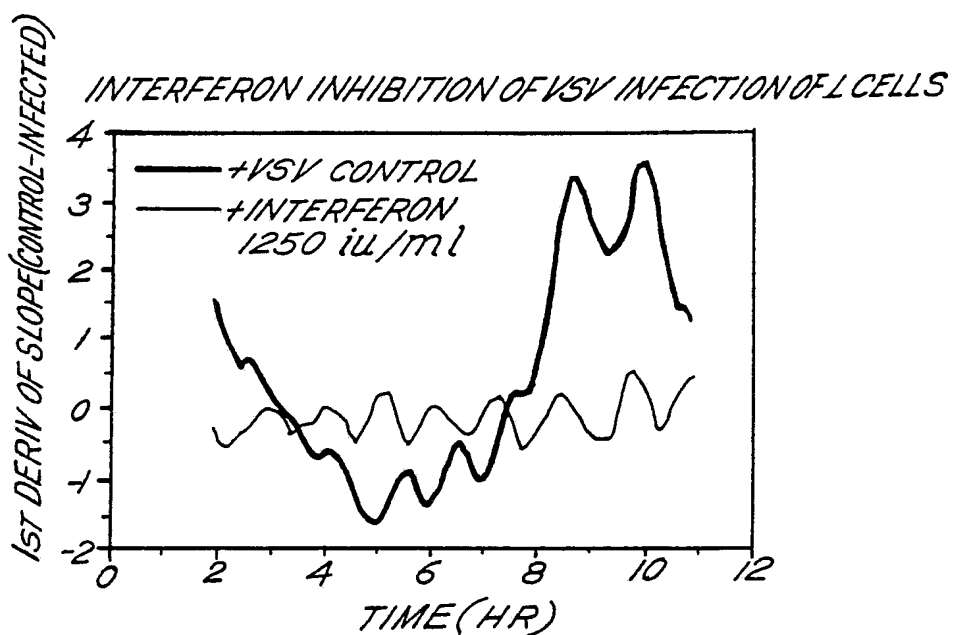
Fig.39

VSV Infection of L Cells

Fig.40
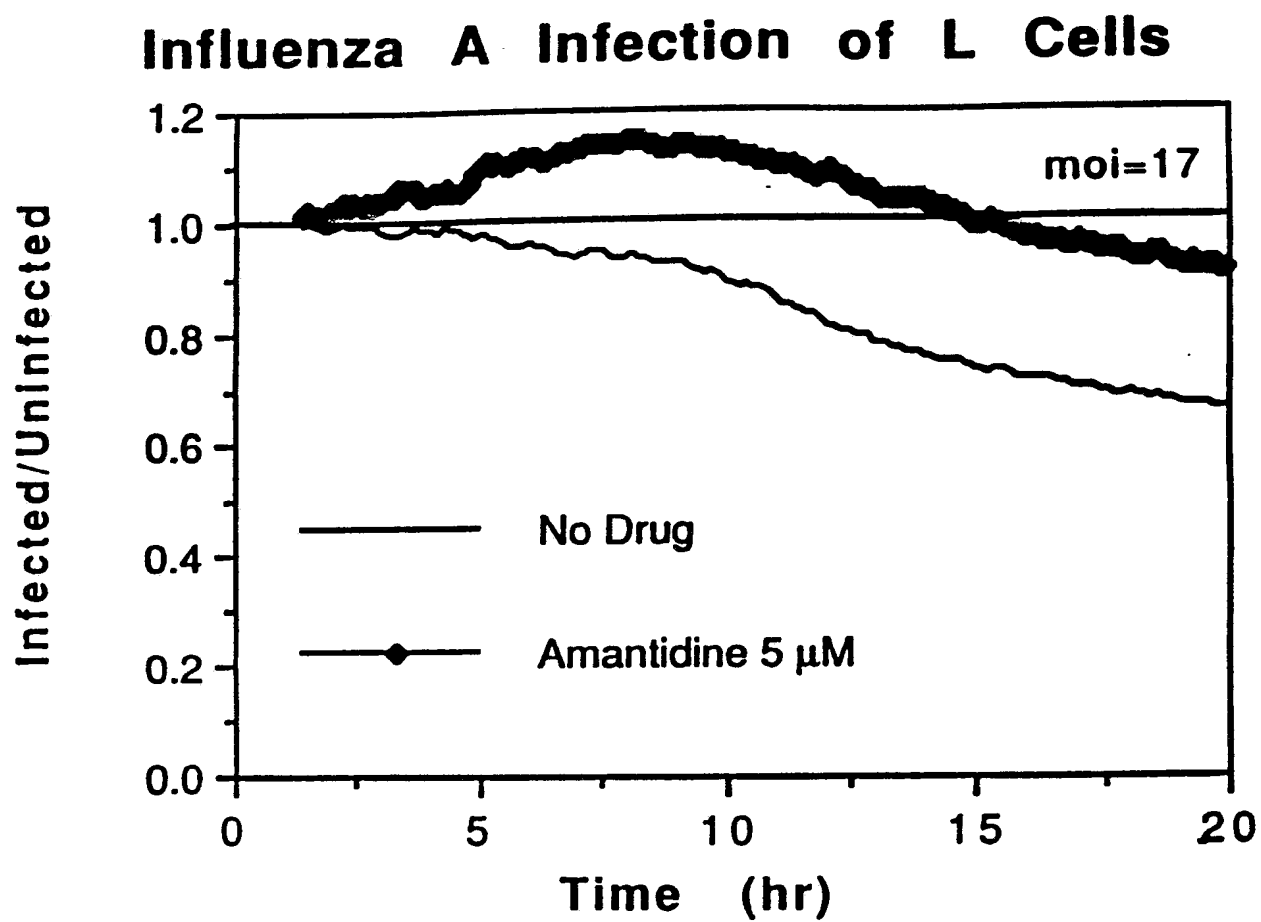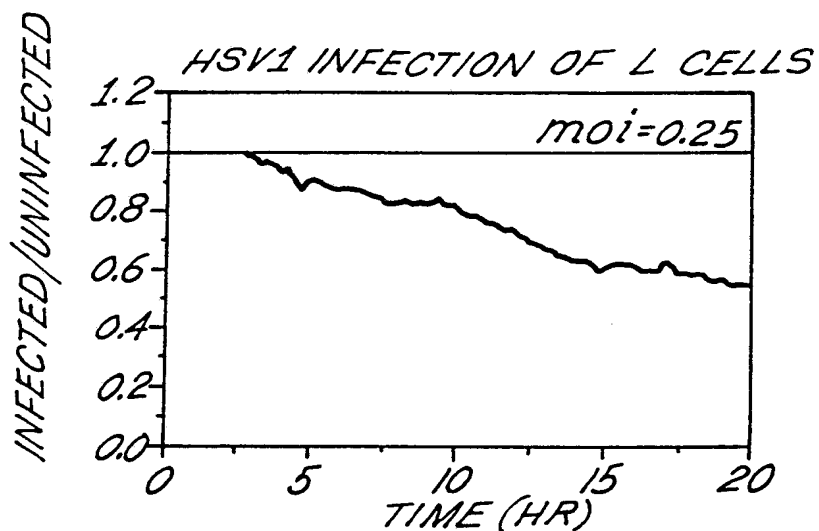
Influenza A Infection of L Cells

moi=17

Infected/Uninfected

No Drug

Amantidine 5 μM

Time (hr)

Fig.41A

HSV1 INFECTION OF L CELLS

moi=0.25

Fig.41B

INFLUENZA A INFECTION OF L CELLS

moi=17

Fig.41C

VSV INFECTION OF L CELLS

moi=1.0
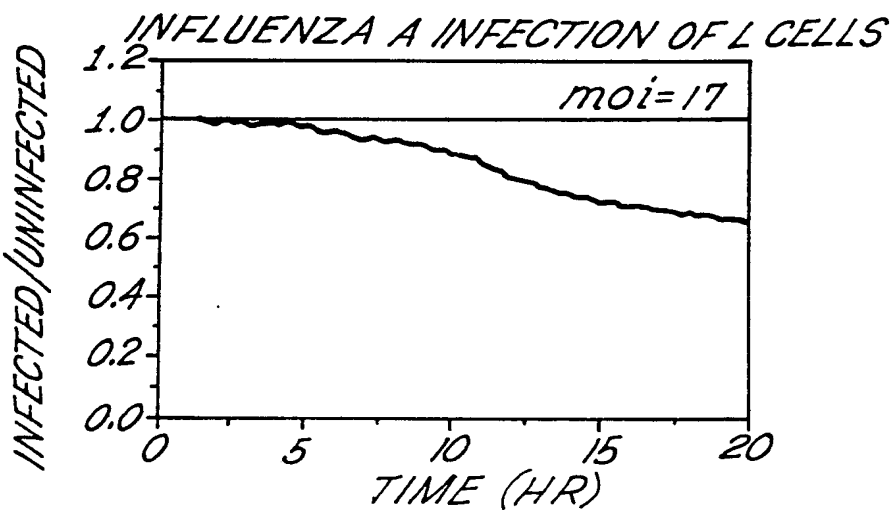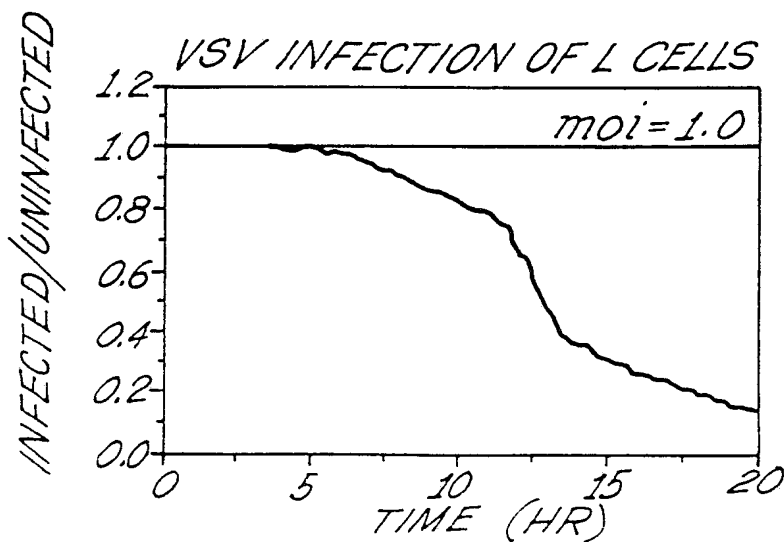
Fig.42
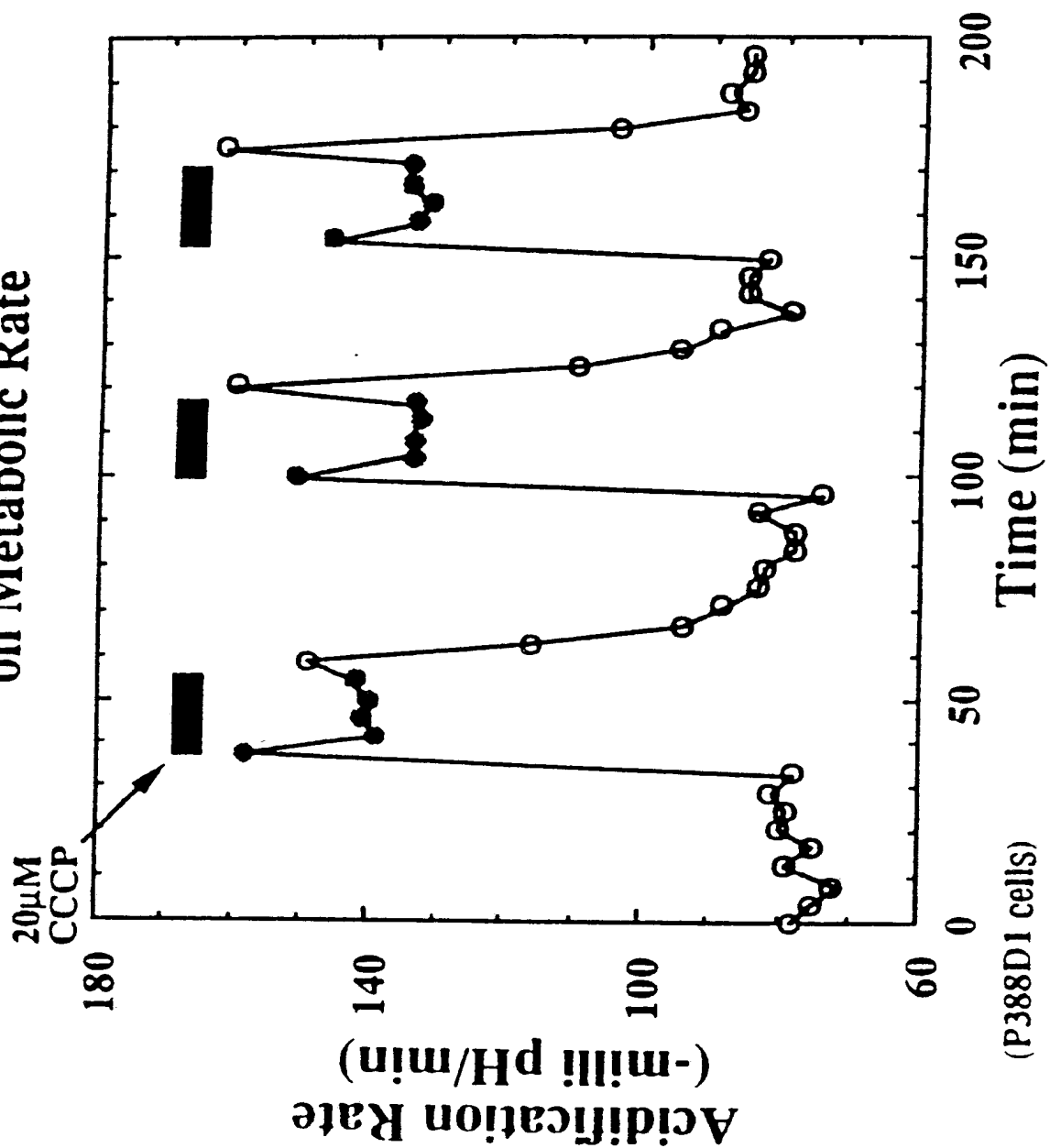
Fig.43

Effect of Epidermal Growth Factor on
Metabolic Rate of Human Keratinocytes

Fig.44

Fig.45

Fig.46

A. 1 µM Doxorubicin

B. 100 µM Vincristine
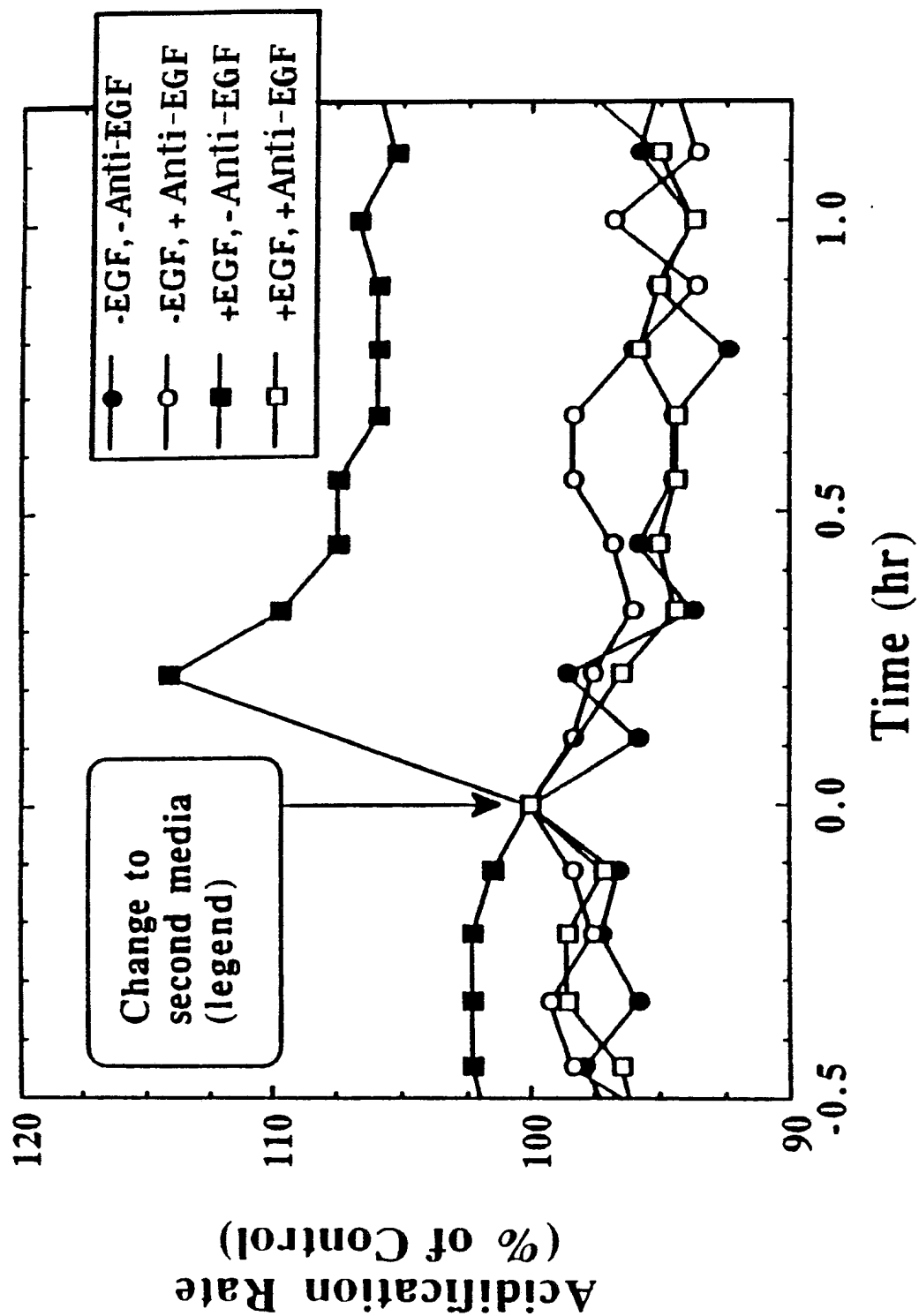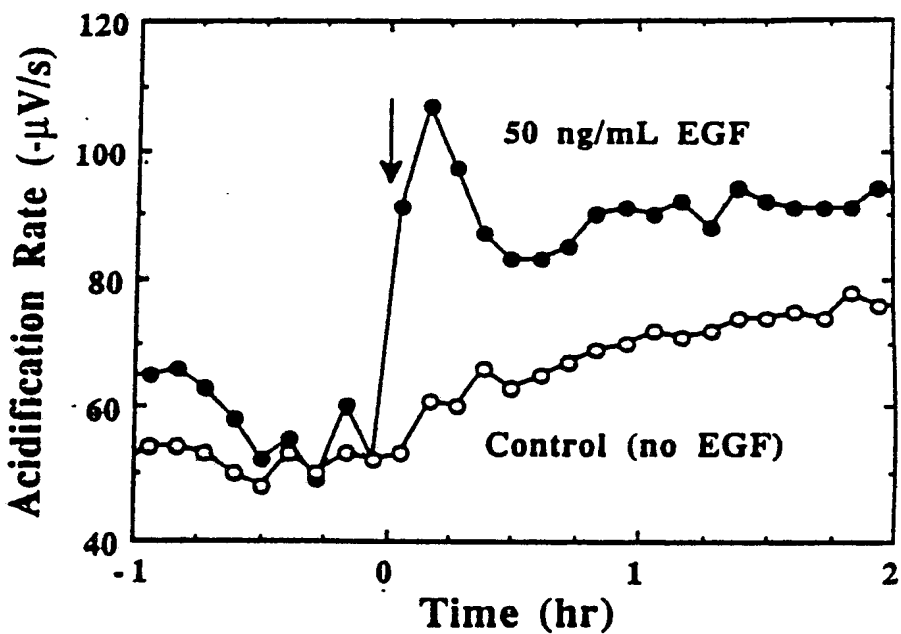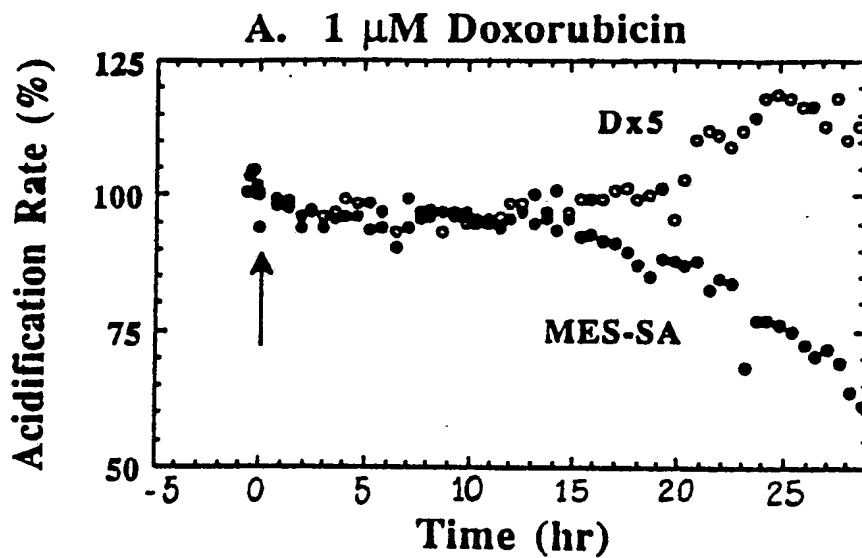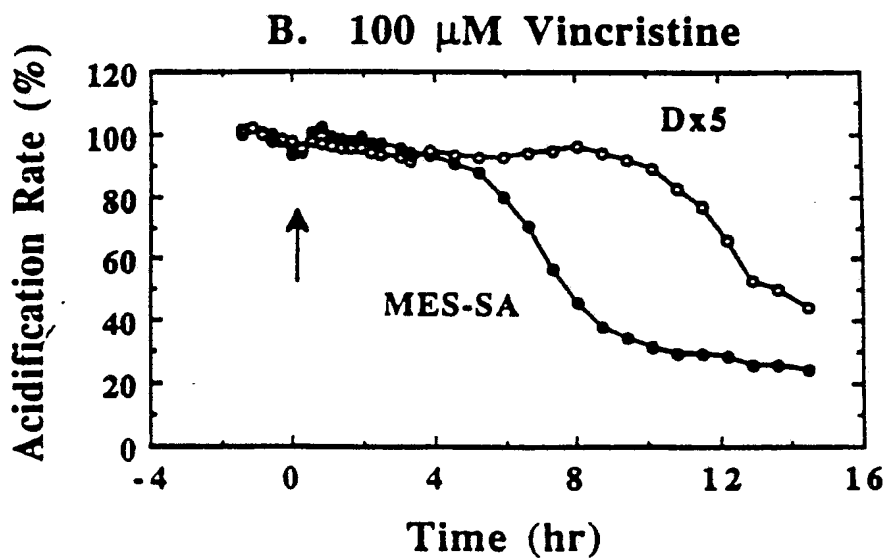
INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/04434

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/02, C 12 M 1/18, 1/40

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC5	C 12 Q; C 12 M

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

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X	EP, A2, 0060509 (PETERSON ANDERS ET AL) 22 September 1982, see the whole document	1,7,19,21, 23,24,25, 26,27,30
Y		1,25
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X	US, A, 4732811 (S MARGEL) 22 March 1988, see the whole document	10-12, 16-18, 37-38
	--	
Y	EP, A2, 0005032 (SHAPIRO, HOWARD MAURICE) 31 October 1979, see claims spec. 4-7	1,25
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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9th January 1990	2 2 JAN. 1990

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	T.K. WILLIS

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III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

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P,X	EP, A2, 0291130 (ENIRICERCHE S.P.A.) 17 November 1988, see the whole document	2-6
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A	Japanese Patents Report, Vol. 82, No. 4, 1982, Derwent Publications LTD:"Incubating living cells - by adhering them to a cationic, semipermeable membrane, and passing incubation liquor on the opposite side", Abstract No D16-J82004235, JP, A, 54028890	8
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